THE UNIVERSITY
of EDINBURGH

Isolation, Characterisation and *In Vitro* Potential of Oogonial Stem Cells

Cheryl Elizabeth Dunlop

BSc Med Sci (Hons), University of Edinburgh

MBChB (Hons), University of Edinburgh

Doctor of Philosophy (PhD) – The University of Edinburgh – 2016

Declaration

This thesis has been composed by myself and the research described herein is my own, except where work by others has been duly acknowledged. The work described in this thesis has not been submitted for any other degree or professional qualification.

Cheryl Dunlop

2016

Abstract

The longstanding belief that women are born with a finite ovarian reserve has been debated for over a decade, ever since the discovery, and subsequent isolation, of purported oogonial stem cells (OSCs) from adult mammalian ovaries. This rare cell population has now been reported in the mouse, rat, pig, rhesus macaque monkey and humans and, although a physiological role for the cells has not been proven, they do appear to generate oocytes when cultured in specific environments, resulting in live offspring in rodents. The primary aim of this thesis was to verify independently the existence of OSCs in human ovary and determine whether they could be isolated from a large animal model, the cow. The secondary aim was to investigate the cells' *in vitro* potential, both to undergo *neo*-oogenesis and as a model for germ cell development.

Putative bovine and human OSCs were isolated from disaggregated adult ovarian cortex using a previously validated fluorescence-activated cell sorting (FACS)-based technique, with cells sorted for externally expressed DDX4 (VASA). Freshly isolated and cultured cells were characterised by analysing their expression of pluripotency and germline markers, using RT-PCR, immunocytochemistry and Western blotting. The *in vitro neo*-oogenesis potential of the cells was explored by injecting fluorescently labelled cells into fragments of adult ovarian cortex and by forming aggregated artificial "ovaries" with putative OSCs and fetal ovarian somatic cells. Germ cell model experiments comprised treatment of cultured cells with BMP4 and/or retinoic acid (RA), with subsequent quantitative RT-PCR and immunocytochemistry analysis for downstream BMP4- and RA-response genes, and liposomal-mediated transfection of cells with a DAZL overexpression plasmid to assess their meiosis-related gene response.

Scarce populations of putative OSCs were retrieved from 5 human samples (aged 13-40 years) and 6 bovine samples. The cells were cultured long-term for up to 7 months and demonstrated consistent expression of several pluripotency-associated and germline markers at the mRNA and protein level, including LIN28, NANOG, POU5F1 (OCT4), IFITM3 (fragilis), STELLA, PRDM1 (BLIMP1), and C-KIT, indicating their early germline nature. Investigation of *neo*-oogenesis potential revealed that putative human OSCs were associated rarely with fetal somatic cells in

primordial follicle-like structures, but could not be confirmed to have undergone oogenesis. However, like early germ cells, putative bovine and human OSCs were BMP4 and RA responsive, with both species demonstrating significant upregulation of expression of *ID1* and bovine cells exhibiting a significant increase in *MSX1*, *MSX2* and the meiotic marker *SYCP3* in response to BMP4 and/or RA treatment. Cells could be successfully transfected to overexpress *DAZL*; however, no significant downstream gene expression changes were observed.

This is the first report of putative bovine OSC isolation and corroborates a previous report showing putative human OSC isolation. Although the expression of both stem cell and germline markers indicates the cells have characteristics of OSCs, their capacity to enter meiosis and form functional oocytes has yet to be determined. Putative bovine OSCs, however, show promise as a novel model for investigating germ cell development. If their potential can be harnessed, then OSCs may have a role in clinical applications, for example in fertility preservation, in the future. Future experiments will examine the *neo*-oogenesis capabilities of the cells further and explore novel cell delivery systems for clinical use.

Lay Summary

It has long been believed that women are born with all the eggs they will ever have. However, since 2004, unique cells have been detected in the ovary that seemingly make new eggs when grown in the right conditions. These cells, called oogonial stem cells (OSCs), have been isolated from mice, rats, pigs, monkeys and humans. When mouse and rat OSCs are transplanted back into ovaries, live mice and rats have been born from eggs apparently created by the stem cells. However, there is no proof that these cells make new eggs under normal circumstances within the ovary and the idea of egg-producing stem cells has been controversial, with many scientists challenging the findings. The aim of this research was to investigate whether these cells exist in cow and human ovaries and, if so, to explore whether they could make new eggs when grown in a dish.

By cutting up pieces of ovary and using an antibody to select cells containing a marker called DDX4, a rare population of cells was isolated from both bovine and human adult ovaries. These cells exhibited markers of a stem cell with egg-like qualities, implying the cells may be able to divide repeatedly over a long period of time and also develop into new eggs. The cells multiplied successfully for up to 7 months in the laboratory and continued to show the stem cell and egg markers. Attempts were made to create eggs from the cells by injecting them back into ovaries and also by creating "artificial ovaries" by combining them with another type of cell that supports egg growth, but it could not be proven that the cells could form new eggs. However, when the cells were treated with two substances (BMP4 and retinoic acid) that encourage the precursors of eggs to mature into eggs before females are born, they behaved in the same way as the egg precursors. This suggested that these cells may provide a great opportunity to look more closely at how eggs grow and develop as they could be used in experiments to simulate what happens to these egg precursors in real life. This is of great scientific value as there is a very limited availability of human eggs to perform experiments on.

This is the first time in the world that these cells have been discovered in the cow and confirms previous research in humans. If these cells can form new, healthy eggs, then they could provide a new treatment option for girls and women at risk of infertility due to cancer treatment who, due to age or time constraints, cannot have eggs collected

prior to starting treatment. Future experiments will explore further the ability of the cells to form new eggs in a dish and also investigate new ways of delivering the cells into ovaries so they can be used in the hospital setting.

Publications relating to this thesis

Published papers

Dunlop CE, Telfer EE, Anderson RA. Ovarian stem cells - potential roles in infertility treatment and fertility preservation. Maturitas. 2013 Nov;76(3):279-83.

Dunlop CE, Telfer EE, Anderson RA. Ovarian germline stem cells. *Stem Cell Res Ther*. 2014 Aug 18;5(4):98.

Dunlop CE, Anderson RA. The regulation and assessment of follicular growth. *Scand J Clin Lab Invest Suppl*. 2014;244:13-7; discussion 17.

Grieve KM, McLaughlin M, **Dunlop CE**, Telfer EE, Anderson RA. The controversial existence and functional potential of oogonial stem cells. Maturitas. 2015 Nov;82(3):278-81.

Full text of first author publications can be found in Appendix 2.

Presentations relating to this thesis

Oral presentations

Human and bovine ovarian cortex contain oogonial stem cells with potential for modelling germ cell development and entry to meiosis.

Dunlop CE, McLaughlin M, Bayne RA, Rosario R, Telfer EE, Anderson RA.

British Fertility Society *Birmingham, UK* *2016*

The isolation, characterisation and *in vitro* culture of ovarian stem cells.

Dunlop CE, McLaughlin M, Bayne RA, Telfer EE, Anderson RA.

Edinburgh Obstetrical Society *Edinburgh, UK* *2013*

Poster presentations

Isolation, propagation and characterisation of oogonial stem cells from human and bovine ovarian cortex.

Dunlop CE, McLaughlin M, Bayne RAL, Anderson RA, Telfer EE.

Society for Reproduction and Fertility *Edinburgh, UK* *2014*

Isolation, propagation and characterisation of oogonial stem cells from human and bovine ovarian cortex.

Telfer EE, **Dunlop CE**, McLaughlin M, Bayne RAL, Anderson RA.

ESHRE Annual Meeting *Munich, Germany* *2014*

Isolation, purification, and culture of oogonial stem cells from adult human and bovine ovarian cortex. [Abstract published in Lancet. 2014 Feb; 383(S45)].

Dunlop CE, Bayne RA, McLaughlin M, Telfer EE, Anderson RA.

Academy of Medical Sciences Spring Meeting *London, UK* *2014*

Isolation, characterisation and *in vitro* culture of oogonial stem cells from adult human and bovine ovarian cortex.

Dunlop CE, Bayne RA, McLaughlin M, Telfer EE, Anderson RA.

RCOG Annual Academic Meeting *London, UK* *2013*

Isolation, purification and culture of oogonial stem cells from adult human and bovine ovarian cortex.

Dunlop CE, Bayne RA, McLaughlin M, Telfer EE, Anderson RA.

Ovarian Club III Meeting *Paris, France* *2013*

Acknowledgements

I am greatly indebted to many people for the help and support required to complete the work contained within this thesis. Firstly, I would like to thank my supervisors, Prof. Richard Anderson and Prof. Evelyn Telfer. Their sage advice, unwavering support and infectious enthusiasm have been much appreciated throughout my PhD. I would like to thank them for giving me the fantastic opportunity to conduct this research and for looking after me so well.

I am equally grateful to my colleagues, now friends, within Prof. Anderson's and Prof. Telfer's groups, who have all contributed in some way to this thesis. Dr. Marie McLaughlin has been a constant source of advice, support (and much appreciated cake!) throughout my time in research. Dr. Rosey Bayne was incredibly patient in teaching me most of the techniques used in this thesis and in answering my many questions. Dr. Roseanne Rosario has been a fantastic teacher and friend who has been incredibly supportive of me and always made coming into the lab fun. Thank you also to Dr. Yvonne Clarkson for helping me with several experimental aspects of this thesis and for her constant encouragement. Kelsey Grieve, my fellow PhD student, has also been a great support to me – thank you for many valuable discussions and for your help in the lab. Both Hazel Kinnell and John Binnie have been fantastic technical support and were always generous with their time. Anne Saunderson and Joan Creiger, Research Nurses, have been a wonderful help by recruiting women for tissue used in this research and were always a friendly ear with a hot cup of tea when required!

Outwith the Anderson and Telfer groups, I need to thank the Flow Cytometry staff for the many hours of work they put in over the years for my research: Fiona Rossi, Will Ramsay and Shonna Johnston at QMRI and Dr. Martin Waterfall at King's Buildings. I am grateful to Dr Pamela Brown and Mike Millar from SuRF at QMRI for their lentivirus and histology expertise respectively. Thanks also to Mr. Luke Davey in the Centre for Inflammation Research for kindly allowing to use some of his equipment and to Charlotte Keith at the South East Scotland Genetics Service for performing chromosomal analysis for me. Prof. Jonathan Tilly and Dr. Dori Woods provided the cell isolation protocol used in this thesis prior to it being published, for which I am

very appreciative. Very importantly, I am extremely grateful to all the women who generously donated their tissue for this research and to my colleagues at Edinburgh Royal Infirmary and St. John's Hospital, Livingston, for collecting it.

The final group of people I need to thank are my family and friends for their unending encouragement and support during my research. In particular, I need to thank my husband, Peter, for pretending to understand what I was talking about when discussing my experiments and for always believing I would finish writing this thesis, even when I didn't believe it myself. Lastly, the work in this thesis was temporarily interrupted by the arrival of our son, Ruairidh. Thank you to him for not minding when I went back to work and for being a constant source of joy, even after a bad day at the bench!

Table of Contents

List of Figures

List of Tables

Commonly Used Abbreviations

ACGC	Adult cortical germ cell
ALP	Alkaline Phosphatase
ANOVA	Analysis Of Variance
APC	Allophycocyanin
ASC	Adipose tissue-derived stem cell
bFGF	Basic Fibroblast Growth Factor
BM	Bone Marrow
β-ME	β-Mercaptoethanol
BMP	Bone Morphogenetic Protein
BrdU	Bromodeoxyuridine
BSA	Bovine Serum Albumin
DAB	3,3'-Diaminobenzidine
DAPI	4',6-Diamidino-2-Phenylindol
DAZL	Deleted in Azoospermia-Like
DDX4	DEAD (Asp-Glu-Ala-Asp) Box Polypeptide 4
DMSO	Dimethyl Sulphoxide
DPBS	Dulbecco’s Phosphate-Buffered Saline
DPPA3	Developmental Pluripotency-associated Protein 3
EB	Embryoid Body
EDTA	Ethylenediaminetetraacetic Acid
ESC	Embryonic Stem Cell
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
fGSC	Female Germline Stem Cell

FISH	Fluorescence *In Situ* Hybridization
FSH	Follicle Stimulating Hormone
GDF-9	Growth Differentiation Factor-9
GFP	Green Fluorescent Protein
HBSS	Hank's Balanced Salt Solution
ICC	Immunocytochemistry
ICM	Inner Cell Mass
IF	Immunofluorescence
IFITM3	Interferon-Induced Transmembrane Protein 3
IHC	Immunohistochemistry
iPSCs	Induced Pluripotent Stem Cells
IVF	*In Vitro* Fertilisation
IVM	*In Vitro* Maturation
LH	Luteinising Hormone
LIF	Leukaemia Inhibitory Factor
MACS	Magnetically-Activated Cell Sorting
MEF	Mouse Embryonic Feeder
MII	Metaphase II
mRNA	Messenger Ribonucleic Acid
MSX	Muscle Segment Homeobox
mtDNA	Mitochondrial Deoxyribonucleic Acid
mw	Molecular Weight
NBF	Neutral Buffered Formulin
NOBOX	Newborn Ovary Homeobox
OLC	Oocyte-Like Cell

OSC	Oogonial Stem Cell
OSE	Ovarian Surface Epithelium
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PFF	Porcine Follicular Fluid
PGC	Primordial Germ Cell
PGCLC	Primordial Germ Cell-Like Cell
PHA	Phytohaemagglutinin
PI	Propidium Iodide
POI	Premature Ovarian Insufficiency
POU5F1	POU Class 5 Homeobox 1
PRDM1	PR Domain Zinc Finger Protein 1
QMRI	Queen's Medical Research Institute
qRT-PCR	Quantitative RT-PCR
RARβ	Retinoic acid receptor beta
RFP	Red fluorescent protein
RPL32	Ribosomal Protein L32
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SBS	School of Biological Sciences
SCF	Stem Cell Factor
S.E.M.	Standard Error of the Mean
STRA8	Stimulated by Retinoic Acid gene 8
STWS	Scott's Tap Water Substitute
SYCP	Synaptonemal Complex Protein
TBS	Tris-Buffered Saline

TGFβ1	Transforming Growth Factor β1
VSEL	Very Small Embryonic-Like
v/v	volume per volume
w/v	weight per volume
YFP	yellow fluorescent protein

Chapter 1

General Introduction

1.1 Development of the Female Germ Cell

1.1.1 Formation of the Ovarian Reserve

In mammals, reproduction is only possible through the production of a unique cell population, known as germ cells. The female and male counterparts of this cell type must become haploid and be able to undergo fertilisation, in order to create a zygote with the correct complement of chromosomes. This process permits perpetuation of the species by giving rise to the next generation. The female germ cell, the oocyte, is the largest cell in the body. It is contained within the fundamental structure of the mammalian ovary, the ovarian follicle, consisting of an oocyte surrounded by supportive somatic (granulosa and theca) cells. The follicle has two essential roles: (i) the maturation and release of an oocyte for fertilisation and (ii) production of reproductive hormones. These hormones, which include estrogen and progesterone, are important for general systemic wellbeing, and are vital to the correct function of several organs in the body including the ovary, uterus, bone and breasts.

The formation of the oocyte population, known as the ovarian reserve, could be considered an extremely wasteful biological process, with millions of female germ cells undergoing attrition during its creation. In mammals, it is a highly regulated process (Fig. 1.1) which starts early in the post-implantation embryo, with paracrine signalling from adjacent cells inducing the specification of a small number of primordial germ cells (PGCs) from a subdivision of epiblast cells within the inner cell mass (ICM) of the blastocyst (Irie *et al.*, 2014). Members of the transforming growth factor β1 (TGFβ1) family called bone morphogenetic proteins (BMPs) appear to be critical for germ cell specification, with mouse studies demonstrating that homozygous null mutants for Bmp4 (Lawson *et al.*, 1999) and Bmp8b (Ying *et al.*, 2000) either have a complete lack of PGCs or at least an extreme depletion. The role of BMP signalling in PGC development will be explored further in Chapter 6.

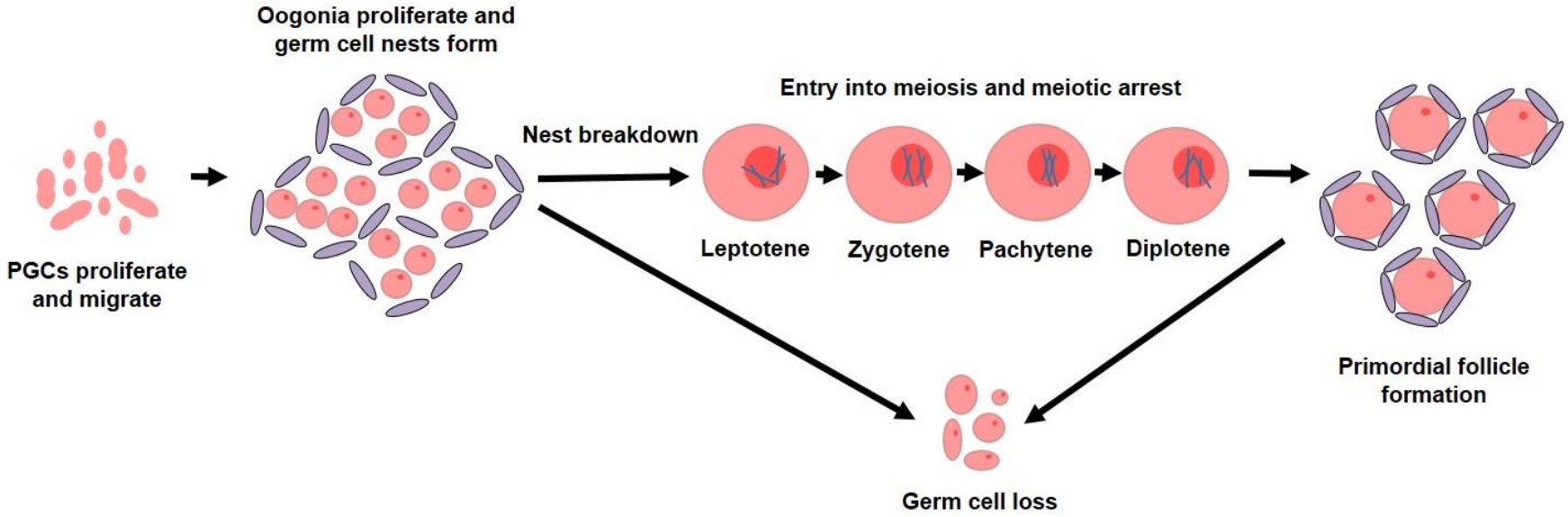


Figure 1.1. Schematic of primordial germ cell (PGC) development. Following specification, PGCs proliferate and migrate to the gonadal ridges before forming germ cell nests. The germ cells then enter meiosis, progressing through the prophase I stages of leptotene, zygotene and pachytene before arresting in diplotene. Nest breakdown allows primordial follicle formation. Throughout the process, germ cell loss occurs due to many different intrinsic and extrinsic factors.

Although PGC development has been most thoroughly studied in mice, this thesis focusses on human and bovine ovarian tissue and therefore the timeline of the process in these two species will be described. Humans and cows have very similar gestation lengths (40 weeks versus 39 weeks) and their ovarian reserves are also formed at comparable timings (Fig. 1.2).

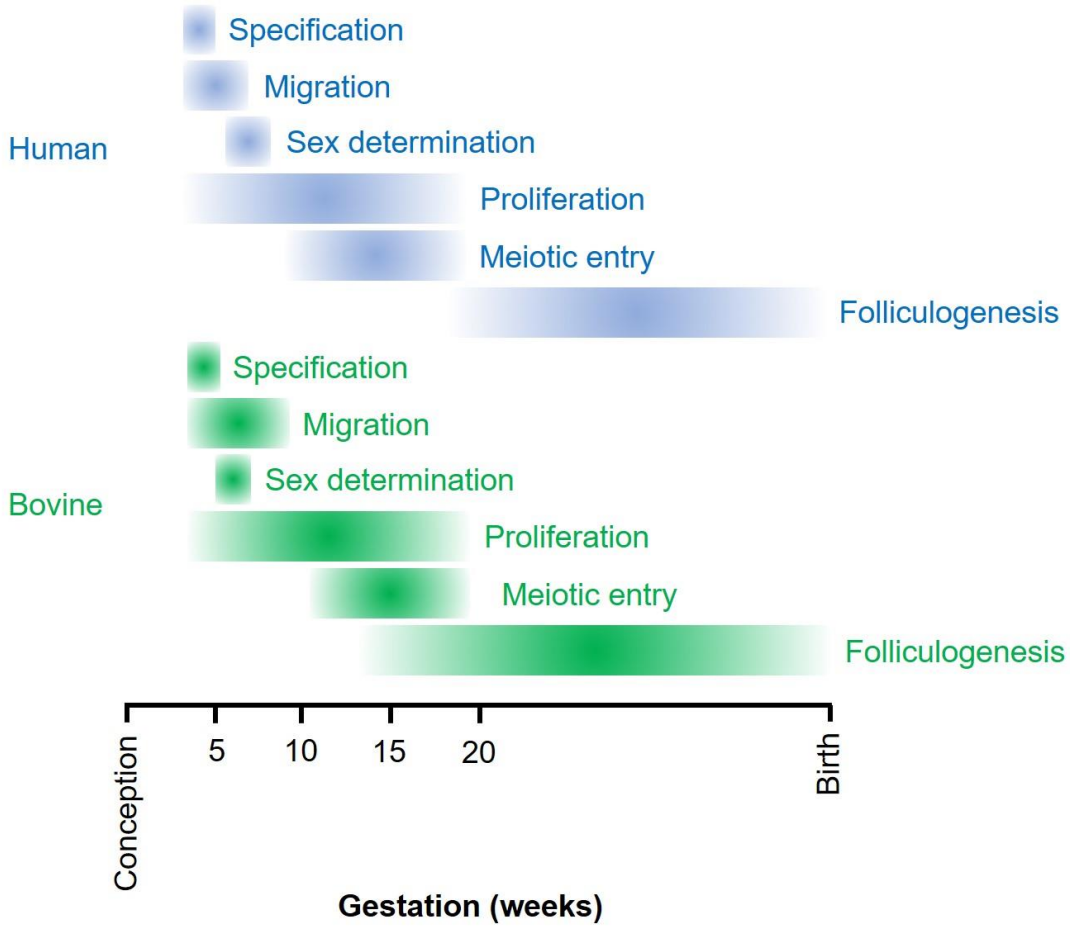


Figure 1.2. Comparison of the time frame for the formation of the follicular pool in the human and bovine fetus, showing very similar temporal processes.

PGCs initially reside within a transient structure known as the primitive streak. From there, rapidly proliferating PGCs migrate to the hind gut using pseudopodia to produce amoeboid movements and subsequently reach the genital ridges by 6 weeks gestation in humans (Fujimoto *et al.*, 1977) and 9 weeks gestation in cattle (Aerts and Bols,

2010). The targeted migration of PGCs appears to be in response to genital ridge-derived chemotactic signals and is aided by cell-cell interactions. The role of the genital ridge in secreting chemoattractants is well established, with mouse PGCs exhibiting migration towards isolated genital ridges *in vitro* (Godin *et al.*, 1990). Both stem cell factor (SCF; also known as Kit ligand, KL) and stromal cell-derived factor 1 (SDF1) have been demonstrated to guide mouse PGC movement: *in vitro* experiments demonstrated that PGCs migrate in response to SCF (Farini *et al.*, 2007), whilst knock-out studies revealed that interaction between genital ridge-derived SDF1 and its receptor, C-X-C chemokine receptor type 4 (CXCR4), on PGCs is required for correct migration to occur (Molyneaux *et al.*, 2003). With regards cell-cell interactions, integrin β1 enables cell migration, with PGC expression of the protein critical for colonisation of the gonads, likely through both cell-cell and cell-ECM interactions (Anderson *et al.*, 1999).

Sexual differentiation occurs at 6-7 weeks gestation in humans (Sarraj and Drummond, 2012) and by 6 weeks in the cow (Erickson, 1966): the absence of the sex-determining region on the Y chromosome (SRY), plus the presence of female sex-determining genes such as wingless-type MMTV integration site family member 4 (WNT4; Vainio *et al.*, 1999), leads to differentiation of PGCs into oogonia and the creation of ovaries (Sarraj and Drummond, 2012). Oogonia continue to undergo mitosis, with numbers in humans increasing from ~26,000 at 6 weeks gestation to ~250,000 at 9 weeks gestation (Bendsen *et al.*, 2006). In cattle, the numbers at equivalent gestations are ~16,000 and ~325,000 respectively (Erickson, 1966). During this time oogonia associate with somatic cells within the gonad, becoming enclosed in germ cell nests. Incomplete cytokinesis during mitotic division of the oogonia results in cytoplasmic bridges connecting the cells, thus allowing bi-directional communication (Pepling and Spradling, 1998, Hartshorne *et al.*, 2009). In cattle, the germ cells are contained within more defined ovigerous cords (Smith *et al.*, 2007).

In order to attain haploid status, germ cells have the unique ability to undergo meiosis. Oogonia enter meiosis from approximately 9-12 weeks gestation in humans (Motta *et al.*, 1997, Bendsen *et al.*, 2006) and 11 weeks in cattle (Erickson, 1966), but entry can continue until around 19 weeks gestation (Hartshorne *et al.*, 2009), meaning that germ cells at many differing stages of maturation can be present in the ovary simultaneously.

There appears to be a spatial distinction between oogonia that are still undergoing mitotic divisions and those entering meiosis, with the former being distributed around the periphery of the ovaries and the latter being located centrally (Anderson *et al.*, 2007). Upon entering meiosis, oogonia are thereafter termed oocytes. Oocytes progress through the stages of prophase I: leptotene (chromatin condensation), zygotene (pairing of homologous chromosomes with formation of synaptonemal complex) and pachytene (recombination of chromosomes) until arresting in the diplotene stage, where chromosomes start to separate from each other, but remain attached at chiasmata (Baillet and Mandon-Pepin, 2012). Mouse studies have demonstrated that the RNA-binding protein Deleted in azoospermia-like (Dazl) is an essential requirement for meiotic entry (Lin *et al.*, 2008) and human studies have shown that DAZL expression is significantly upregulated around the time of meiotic entry, with its expression being centrally located in "nests" (Anderson *et al.*, 2007). It is possible that the synaptonemal complex protein 3 (*SYCP3*) gene, which is required for the pairing of homologous chromosomes in zygotene (Yuan *et al.*, 2002), is a DAZL target in the human female gonad as male knock-out mice studies have demonstrated that Dazl increases the translation of *Sycp3* mRNA (Reynolds *et al.*, 2007). Ovarian-derived retinoic acid (RA) is also regarded as critical in inducing meiotic entry in mammals, including humans (Le Bouffant *et al.*, 2010, Childs *et al.*, 2011), acting through Stimulated by retinoic acid gene 8 (*STRA8*). STRA8 is a key regulator of the initiation of meiosis, with *Stra8*-deficient mouse PGCs unable to undergo premeiotic DNA replication and subsequently failing to demonstrate the chromosomal changes required during meiosis (Baltus *et al.*, 2006). The roles of both DAZL and RA in meiosis will be discussed in more depth in Chapter 6.

After approximately 18 weeks gestation in humans, germ cell nests begin to break down and the earliest follicular structures, called primordial follicles, are formed. The first primordial follicles in bovine ovaries can be seen by 13 weeks gestation (Yang and Fortune, 2008). In mice, it has been postulated that nest breakdown occurs in a stepwise manner due to apoptosis of some of the oogonia within the nests: this creates smaller nests which continue to decrease in size due to continuing apoptosis, until a small number of oocytes remain which will form primordial follicles (Pepling and Spradling, 2001). In this model, the oogonia that undergo apoptosis are considered

"nurse cells", supplying essential molecules via the cytoplasmic bridges to the neighbouring oogonia that will ultimately become oocytes, before undergoing programmed cell death (Pepling and Spradling, 2001). There is no evidence supporting the extension of this theory to humans or cattle, however. Primordial follicles consist of an oocyte arrested in prophase I, surrounded by one layer of flattened squamous cells and arise when pre-granulosa cells are recruited by oocytes. Concurrently, PGCs continue to proliferate, with the number of resultant oogonia reaching a peak of ~5 - 7 million by the 5th month of gestation in humans (Hartshorne *et al.*, 2009) and ~2.7 million by the 4th month of gestation in cattle (Erickson, 1966). Subsequently, however, there is substantial germ cell elimination: in addition to apoptosis of oogonia within nests, death occurs in non-enclosed oogonia/oocytes. The exact reason for this germ cell loss is unknown, but may be due to intrinsic factors, such as genetic errors generated during prophase I or failure to arrest, and/or extrinsic factors, including erroneous paracrine signalling from neighbouring somatic cells with the absence of "survival factors" such as activin (Hartshorne *et al.*, 2009). Moreover, research suggests that the B-cell lymphoma/leukaemia 2 (BCL2) family of proteins, which comprises both pro- and anti-apoptotic members, play a key role in germ cell death (Aitken *et al.*, 2011, Hutt, 2015). Examples include the pro-apoptotic BCL-2 associated X protein (BAX) and the anti-apoptotic BCL2 protein. Bax is upregulated in fetal mouse oocytes undergoing apoptosis (DeFelici *et al.*, 1999), with knock-out studies demonstrating that Bax-deficient mice have increased numbers of oocytes (Greenfeld *et al.*, 2007). In contrast, Bcl2-deficient mice exhibited significantly reduced oocyte numbers (Ratts *et al.*, 1995).

Oocyte atresia is so extensive that, in the human, only ~600,000 follicles are formed from ~5 - 7 million germ cells by the 5th month of gestation, with ~590,000 follicles remaining by the time of birth, and there are merely ~360,000 still present by 13 years of age (Wallace and Kelsey, 2010). In cattle, less than 70,000 follicles remain at birth (Erickson, 1966). During reproductive life, oocytes within primordial follicles remain quiescent, arrested in the diplotene stage of prophase I, until they are activated to grow. Cohorts of immature follicles are continuously recruited, with one follicle selected for ovulation per menstrual cycle. This contributes to the inexorable decline of follicles

until the ovarian reserve reaches approximately 1000 follicles, at which point the menopause occurs (Fig. 1.3).

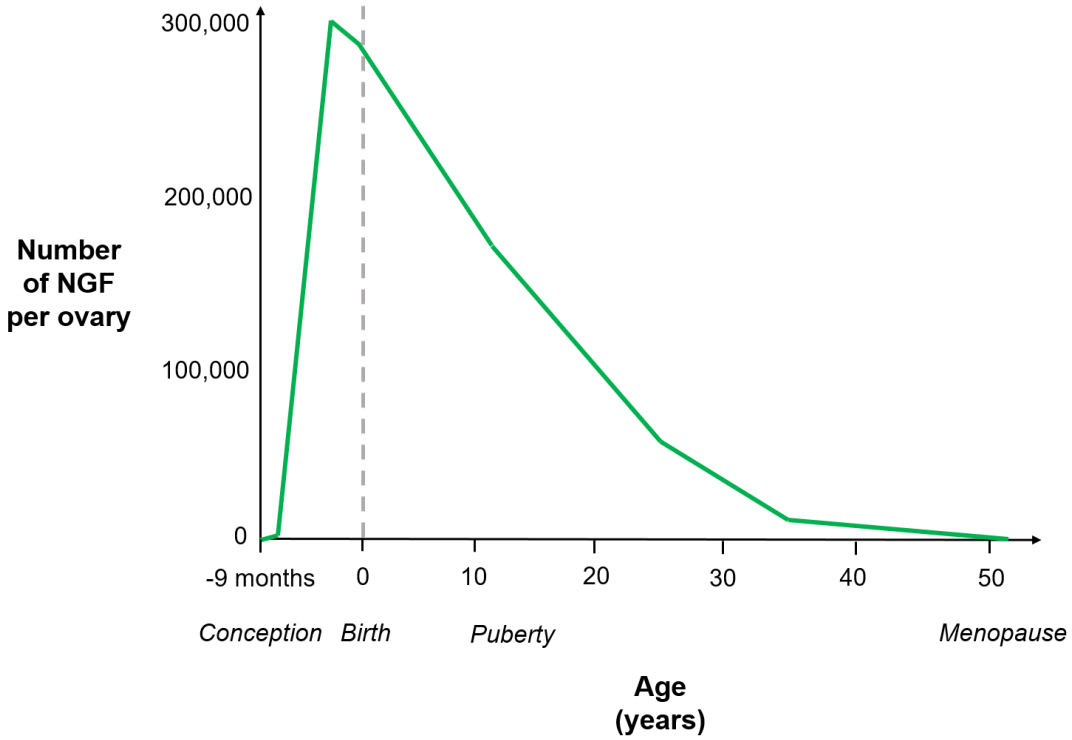


Figure 1.3 The change in non-growing follicle (NGF) numbers per ovary from conception to menopause in humans. Follicles peak in numbers at approximately 5 months gestation before declining throughout life. The decline is initially rapid, but slows in later reproductive years. Exhaustion of the ovarian reserve results in the menopause. Based on data from Wallace and Kelsey, 2010.

1.1.2 Does post-natal oocyte formation occur?

The current well-established and widespread belief within the field of Reproductive Biology is that women possess their lifetime's supply of oocytes by the time they are born, known as a fixed ovarian reserve, and that the menopause occurs once this supply has been exhausted. However, the debate surrounding whether or not female mammals are furnished with a fixed quantity of germ cells at birth has been ongoing since the 1800s (Fig. 1.4).

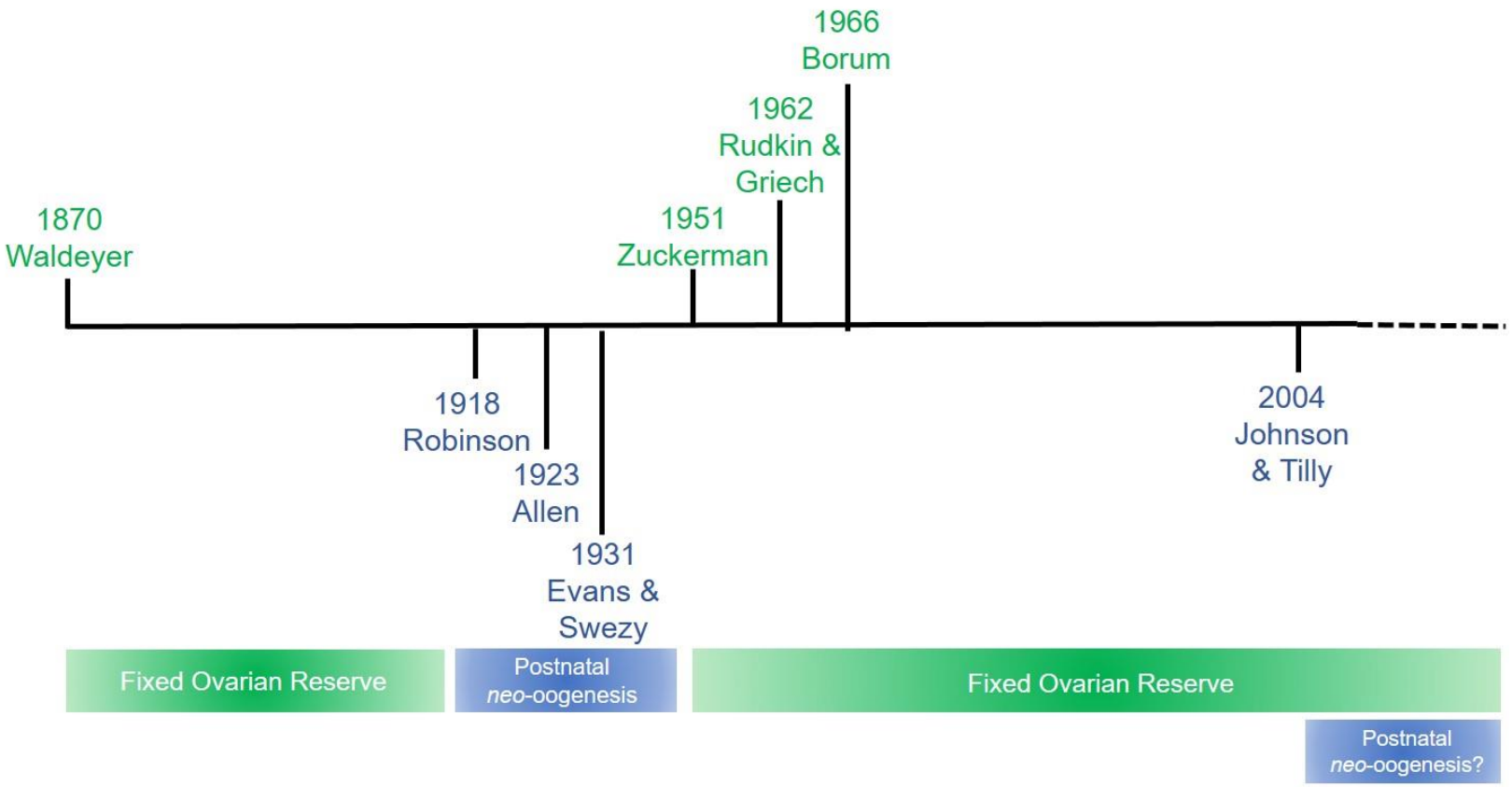


Figure 1.4. An abridged history of the debate surrounding the current dogma of a fixed ovarian reserve. Proponents for the doctrine are in green and supporters of the theory of post-natal *neo*-oogenesis are in blue. The debate was reignited in 2004 by Jonathan Tilly's group (Johnson *et al*., 2004) and is ongoing.

Waldeyer, a 19th century embryologist, is widely regarded as being the first advocate for the idea that females are born with a finite supply of oocytes (Waldeyer, 1870) and his views were supported by several other well-known biologists in the early 1900s (Allen, 1923). However, the prevailing belief began to shift in the 1920s, when proponents for the theory that *neo*-oogenesis occurs in the post-natal ovary emerged, with their assertions based on thorough histological analysis of ovarian tissue (Robinson, 1918, Allen, 1923, Evans and Swezy, 1932). Indeed, following investigations in rats, guinea pigs, cats, dogs and humans, Evans and Swezy stated firmly that:

> "The concept that…the ova are all formed before birth and remain quiescent until sexual maturity calls them into activity, has no foundation in fact. On the contrary, all the ova of adult life are new formations and are being constantly produced and as constantly destroyed." (Evans and Swezy, 1932)

It wasn't until the landmark paper of Sir Solomon Zuckerman in 1951 that the question appeared to be answered once and for all (Zuckerman, 1951). Following his extensive review of the existing literature involving oocyte counting, he concluded, in direct contrast to Evans & Swezy (Evans and Swezy, 1932):

> "None of the experimental and quantitative evidence which we have considered thus supports the view that oogenesis occurs in the adult ovary, and much of it bears very clearly against the proposition." (Zuckerman, 1951)

The evidence presented by Zuckerman was such that he established the basic doctrine that has been steadfastly upheld ever since: female mammals form no new germ cells post-natally (Zuckerman, 1951). This is one of the most significant examples of sexual dimorphism in mammals as it is in direct contrast to the male germ cell population, which undergoes continuous proliferation throughout post-pubertal life. The dogma was subsequently supported by studies utilising tritiated thymidine labelling of proliferating cells, which failed to demonstrate the presence of mitotically active germ cells by monitoring the uptake of the radioisotope in the ovaries of adult mice (Rudkin and Griech, 1962, Borum, 1967) and rats (Hirshfield, 1984). Furthermore, treatment of rats *in utero* with busulphan, a chemotherapeutic agent that damages PGCs, was shown to cause premature exhaustion of the ovarian reserve (Hirshfield, 1994). This appeared to indicate that the ovarian reserve is determined prior to birth, with no *neo*-oogenesis occurring post-natally, although a deleterious effect of busulphan on the

ovarian stromal cells could have contributed to the reduction in follicle formation. Henderson and Edwards' "production line hypothesis" is also regarded as supportive of the concept of a fixed ovarian reserve: analysis of the frequency of chiasmata within mouse oocytes revealed that frequency decreased with increasing age of the mouse (Henderson and Edwards, 1968). It was hypothesised that this indicated oocytes recommence meiosis in the order they are formed in the fetal gonad, with oocytes in older mice undergoing more proliferation pre-natally than younger oocytes. If post-natal *neo*-oogenesis occurs then, according to this theory, it could be assumed that little change in chiasmata frequency would be seen with age as newly formed oocytes in the older mice would not exhibit low chiasmata frequencies. However, the "production line hypothesis" remains a theory; indeed, a recent study of chiasmata frequency in prophase I-stage oocytes of human fetal ovaries demonstrated no correlation between levels of recombination and the gestation at which the oocytes entered meiosis (Rowsey *et al.*, 2014).

This central tenet of the field was not called into question again until 2004, when Johnson *et al*. reported the existence of what have been termed female germline stem cells (fGSCs) in the adult mouse ovary which appeared capable of forming new oocytes post-natally (Johnson *et al.*, 2004). Subsequently, the debate has been reignited, with proponents for and against the theory of *neo*-oogenesis (Powell, 2007) (discussed in depth in section 1.2).

1.1.3 Ovarian Insufficiency

The fixed ovarian reserve dogma may once again be up for debate, however, what is unquestionable is that reproductive senescence occurs. Ovarian insufficiency, where the ovary can no longer maintain its reproductive and endocrinological roles, is a physiological, age-related process occurring at an average age of 51 years old in women (Nelson *et al.*, 2013). With increasing life expectancies, the length of a woman's post-reproductive lifespan is becoming longer, distinguishing humans from other animals, including primates (Hawkes *et al.*, 1998). In addition to troublesome menopausal symptoms such as hot flushes and night sweats that affect quality of life, post-menopausal loss of ovarian-derived estradiol has significant effects on women's health, with issues including osteoporosis, heart disease and depression (Sowers and La Pietra, 1995).

There are also pathological causes of premature ovarian insufficiency (POI) which affect younger women and girls. POI has a heterogeneous aetiology but can be broadly categorised as either iatrogenic (i.e. caused by medical treatment) or non-iatrogenic. Overall, idiopathic POI accounts for the majority of POI cases (Goswami and Conway, 2007); however, increasing numbers of girls and young women of reproductive age are suffering iatrogenic POI as a result of life-saving treatment for diseases such as cancer and autoimmune diseases (Panay and Kalu, 2009). Improvements in such treatments have led to a significant increase in survival rates over the last 15-20 years, especially in childhood cancers (Magnani *et al.*, 2006), hence a preponderance of POI sufferers who have not yet started, or completed, their family.

Although it is becoming clear that cancer treatment can have wide-reaching long-term adverse effects, it is the irreversibility of POI (due to the assumed inability to make new oocytes post-natally) that can be one of the most upsetting consequences, with sufferers reporting a lower quality of life than those cancer survivors without POI (Kondapalli *et al.*, 2014). Current fertility preservation strategies are becoming more effective; however, an ideal fertility preservation strategy for these women would not only prevent a delay in life-saving treatment but also produce a large number of oocytes. The idea that fGSCs may exist and could prove useful in this regard is one of the principal reasons for research into their existence and function.

1.2 Germline Stem Cells

1.2.1 The Function of Germline Stem Cells

Stem cells are defined by their ability to self-renew and differentiate into different cell types. Adult stem cells have a more restricted lineage than embryonic stem cells (ESCs) but are able to proliferate for protracted periods of time and their differentiated progeny are essential for the ongoing maintenance and repair of tissue over an animal's lifespan. This includes sustaining the circulating blood cell populations and allowing turnover of intestinal luminal cells. Germline stem cells (GSCs) are a unique type of unipotent adult stem cell as they differentiate into the gametes that are vital for the continuation of a species.

The concept of a GSC was first hypothesised in 1901 during studies on spermatogenesis (Regaud, 1901a, Regaud, 1901b). The author proposed the theory that the post-pubertal testis must contain a population of cells that could both self-

renew and differentiate into mature sperm. Decades of subsequent study have validated this hypothesis and male GSCs, known as spermatogonial stem cells (SSCs), have been recognised to participate in spermatogenesis in all species in which the process has been studied (Brinster, 2007). Research into the equivalent cell type in females has produced more conflicting data, with fGSCs having a confirmed role in post-natal *neo*-oogenesis in some species (discussed further in section 1.2.3).

Equally as important as the GSCs themselves is their surrounding microenvironment, known as the germ cell niche. These specialised somatic areas within the gonad are responsible for controlling the self-renewal and differentiation of GSCs, thus maintaining the population (Spradling *et al.*, 2011). Furthermore, it is thought that it is the presence of germ cell niches that differentiates those species that undergo post-natal *neo*-oogenesis from those that cannot (Spradling *et al.*, 2011). The exact mechanisms by which the niche "holds" GSCs in an undifferentiated state have not been elucidated, but bi-directional paracrine and juxtacrine signaling between the somatic cells and GSCs likely plays a critical role. In mammals, communication between daughter germ cells is also possible due to the cytoplasmic bridges formed between the cells during incomplete mitotic cytokinesis of the founder cell. These bridges are large, measuring 0.5 - 3µm, and can allow both molecules and organelles as large as mitochondria to transfer between cells (Greenbaum *et al.*, 2011). Such "cytoplasmic sharing" is hypothesised to regulate the synchronisation of mitosis and control entry into meiosis (Greenbaum *et al.*, 2011). The germ cell niche has been investigated in detail in both *Drosophila* and the nematode, *Caenorhabditis elegans* (*C. elegans*) and will be discussed in more detail in section 1.2.3.

1.2.2 Prospective Molecular Markers of Mammalian GSCs

If a cell is to be denoted a GSC, it would be assumed to express both markers of stemness and of the germline, thus indicating its early germline nature. Detailed in this section are some candidate molecular markers of fGSCs based upon their expression in stem cells and/or germ cells. A summary of the markers is presented in Table 1.1.

Table 1.1. Summary of prospective female germline stem cell markers. The presence (green tick) or absence (red cross) of expression of each marker at different stages of development is shown. Conflicting data on expression is illustrated by a question mark. *DPPA3* and *PRDM1* are only expressed at low levels in ESCs, therefore are not considered solely as germ cell markers.

Gene	Type of Marker	Expression at Developmental Stage					
		Embryonic Stem Cells	Pre-Migratory PGCs	Migratory PGCs	Post-Migratory PGCs	Pre-meiotic Oogonia	Meiotic Oocytes
POU5F1	Pluripotency and Germ Cell	✓	✓	✓	✓	✓	X
LIN28	Pluripotency and Germ Cell	✓	✓	✓	✓	✓	X
NANOG	Pluripotency and Germ Cell	✓	✓	✓	✓	✓	X
PRDM1	Germ Cell	✓	✓	✓	✓	✓	X
DPPA3	Germ Cell	✓	✓	✓	✓	✓	✓
IFITM3	Germ Cell	X	✓	?	✓	✓	✓
C-KIT	Germ Cell	X	✓	✓	✓	✓	✓
DDX4	Germ Cell	X	✓	✓	✓	✓	✓

1.2.2.1 POU5F1

POU Class 5 Homeobox 1 (POU5F1), otherwise known as octamer-binding transcription factor 4 (OCT4) is a transcription factor of the Pit-Oct-Unc (POU) family whose gene is highly conserved between mouse, cows and humans (Pesce *et al.*, 1998). Its expression in both mammalian ESCs and germ cells is well established (Okamoto *et al.*, 1990, Rosner *et al.*, 1990, Nichols *et al.*, 1998, Pesce *et al.*, 1998, Richards *et al.*, 2004): it appears to prevent differentiation of stem cells, with its expression being downregulated as cells proceed along a differentiation pathway. It can be found in murine embryos as early as the 4-cell stage and is subsequently localised to the epiblast of the ICM (Pesce *et al.*, 1998). Its importance in early embryogenesis has been demonstrated by the peri-implantation lethality of the homozygous knock-out genotype in mouse embryos due to lack of pluripotency of the ICM (Nichols *et al.*, 1998). Furthermore, both human (Yu *et al.*, 2007) and bovine (Nong *et al.*, 2015) embryonic fibroblasts have been reprogrammed to become induced pluripotent stem cells (iPSCs) by introducing pluripotency genes, including *POU5F1*, into the differentiated cells.

As gastrulation occurs, POU5F1 expression becomes constrained to the germline until it is specifically located in PGCs. Murine studies targeting post-implantation POU5F1 expression by using a Cre/loxP system specific to PGCs have revealed that lack of POU5F1 leads to PGC apoptosis rather than differentiation (Kehler *et al.*, 2004), indicating that it is essential for PGC survival, but not for maintaining PGCs in their dedifferentiated state. Furthermore, it has also been demonstrated to be critical for PGC specification (Okamura *et al.*, 2008). In mice, once PGCs have entered meiosis, POU5F1 is downregulated (Pesce *et al.*, 1998). Its expression is switched back on again just after birth in meiotically-arrested oocytes and persists until the late stages of oocyte maturation (Pesce *et al.*, 1998). In contrast, human POU5F1 expression decreases over time: it is highly expressed in PGCs, but declines during the second trimester with expression limited to mitotic cortical germ cells and disappears when oocytes are undergoing folliculogenesis (Stoop *et al.*, 2005, Anderson *et al.*, 2007). This suggests that once oocytes are in close contact with granulosa cells and are undergoing meiosis, they are no longer capable of being pluripotent. In summary, given its presence in both stem cells and PGCs, POU5F1 seems an appropriate marker for a fGSC.

1.2.2.2 LIN28

Similarly to POU5F1, LIN28 is a candidate marker of fGSCs as it is found in both stem cells and the germline. It is a gene of ancient origin, with homologues in many different species including *Drosophila*, mice and humans (Moss and Tang, 2003). It encodes for a RNA-binding protein initially discovered to be involved in the timing of embryonic development of *C. elegans* (Ambros, 2000). It was then discovered to be widely expressed in the developing embryos of other animals, but had more limited expression in differentiated adult cells (Moss and Tang, 2003). Subsequent studies in human ESCs (hESCs) demonstrated that LIN28 expression was downregulated as hESCs differentiated and suggested, therefore, that LIN28 could be used as a marker of stemness (Richards *et al.*, 2004). As with POU5F1, both human (Yu *et al.*, 2007) and bovine (Nong *et al.*, 2015) embryonic fibroblasts could be reprogrammed to iPSCs by *LIN28*.

The role of LIN28 within the germline has been described with changing temporal and spatial expression over the course of the first and second trimesters in fetal human ovaries (Childs *et al.*, 2012). Its expression is restricted to PGCs and is highest in the first trimester, with all PGCs expressing the protein. In the second trimester, only immature germ cells around the periphery of the ovary continue to express LIN28, with no expression detected in the more centrally located mature germ cells. Furthermore, LIN28-positive germ cells co-expressed POU5F1 but never demonstrated expression of the meiotic entry marker, SYCP3, indicating it is an early PGC marker (Childs *et al.*, 2012). It has been suggested that LIN28 performs its pluripotency role by inhibiting the transcriptional processing of a microRNA (pri-let-7 miRNA) which is important in cell differentiation (Viswanathan *et al.*, 2008). The similarities in temporal expression of LIN28 and its miRNA target across gestations in human fetal ovaries further supports that their functions are connected (Childs *et al.*, 2012).

1.2.2.3 NANOG

NANOG is another transcription factor expressed in both pluripotent stem cells and germ cells, thus rendering it a prospective marker of fGSCs. It is found in both mouse

(Chambers *et al.*, 2003, Mitsui *et al.*, 2003) and human (Chambers *et al.*, 2003, Richards *et al.*, 2004) ESCs and, in conjunction with POU5F1, is critical to maintaining the cells in a pluripotent state of self-renewal with downregulation of NANOG coinciding with increased cell differentiation (Chambers *et al.*, 2003, Clark *et al.*, 2004). Its role in generating iPSCs from human (Yu *et al.*, 2007) and bovine (Nong *et al.*, 2015) fetal fibroblasts has also been demonstrated.

In the human embryo, NANOG expression is subsequently restricted to PGCs within the fetal ovary (Perrett *et al.*, 2008) and in mice, it is expressed in proliferating and migrating PGCs until the time of meiotic entry (Chambers *et al.*, 2003, Yamaguchi *et al.*, 2005). It has been shown to be essential to migrating PGC survival in the mouse embryo, with inducible, specific knockdown of Nanog in PGCs of transgenic mice resulting in migratory PGC apoptosis and not differentiation (Yamaguchi *et al.*, 2009). Yamaguchi *et al*. postulated that the cell death may be a result of downstream gene dysregulation, with Nanog-deficient PGCs demonstrating downregulation of genes including the DNA-binding protein inhibitor, *Id1* (Yamaguchi *et al.*, 2009). Therefore, NANOG is a marker of both ESCs and early, pre-meiotic PGCs.

1.2.2.4 PRDM1

PR domain zinc finger protein 1 (PRDM1; also known as BLIMP-1) is a transcriptional repressor expressed in all three layers of the blastocyst (ectoderm, endoderm and mesoderm), including the primitive streak (Ohinata *et al.*, 2009) and in migratory PGCs (Chang *et al.*, 2002). Its structure includes zinc fingers for DNA binding (Saiti and Lacham-Kaplan, 2007). Lineage tracing using a *Prdm1-Cre-GFP* transgenic mouse strain demonstrated that it is only found in cells destined to be PGCs and its expression in PGCs appears to precede both Developmental pluripotency-associated protein 3 (Dppa3) and Interferon-induced transmembrane protein 3 (Ifitm3) expression thereby making it the earliest marker of mouse PGCs (Saiti and Lacham-Kaplan, 2007, Ohinata *et al.*, 2009). Furthermore, *Prdm1*-mutant mice have a reduced number of PGCs which neither proliferate nor migrate correctly, indicating the critical role of the protein in PGC development (Vincent *et al.*, 2005, Ohinata *et al.*, 2009). Bmp4 (which, as mentioned in section 1.1.1, is necessary for germ cell specification)

has been shown to be important in the induction of epiblast cells to express *Prdm1* and thus facilitates germ cell specification (Ohinata *et al.*, 2009).

In humans, PRDM1 expression has also been demonstrated in fetal ovaries, whilst hESCs upregulate its expression during differentiation into germ cells (Lin *et al.*, 2014). Chronologically, it appears to be expressed at the same stage of differentiation as POU5F1, but prior to DEAD (Asp-Glu-Ala-Asp) box polypeptide 4 (DDX4) and SYCP3 (Lin *et al.*, 2014), indicating it may be a premeiotic marker of germ cells. Data on the role of PRDM1 in bovine germ cell development are not available. Of note, although PRDM1 is clearly a marker of PGCs, it has many other roles throughout the body, including differentiation of B lymphocytes (Chang *et al.*, 2002), therefore it cannot be considered a germ cell-specific marker.

1.2.2.5 DPPA3

DPPA3 has several aliases, including STELLA, STELLAR and PGC7. It was initially detected in preimplantation embryos, PGCs and oocytes of all stages of development in the mouse and deemed to be an excellent marker of cells of early germline lineage (Saitou *et al.*, 2002). More precisely, in the post-implantation embryo it is expressed from the time of PGC specification through PGC migration until embryonic day 13.5 (E13.5) whereupon it is downregulated until birth, when immature oocytes begin to express it again (Saitou *et al.*, 2002). Despite the timing of the onset of its expression, a knock-out study demonstrated that it is not required for germ cell specification (Bortvin *et al.*, 2004). As it is not expressed in mature sperm (Saitou *et al.*, 2002), Dppa3 is therefore a maternal factor of embryogenesis and it has been shown to be essential in protecting the early mouse embryo from premature DNA methylation (Nakamura *et al.*, 2007). As such, it allows correct genomic imprinting and epigenetic modifications which are critical for normal development.

Its human homologue is expressed on chromosome 12 (Bowles *et al.*, 2003) and, although it is expressed at low levels in hESCs, its expression is upregulated in hESCs undergoing germ cell differentiation (Wongtrakoongate *et al.*, 2013). Expression is restricted to the fetal ovary by the second half of pregnancy (Clark *et al.*, 2004) and it appears to be expressed at the same stage of differentiation as POU5F1 and PRDM1

(Lin *et al.*, 2014). Post-natally, both primordial follicles and mature, metaphase II (MII) oocytes express DPPA3 in humans (Grondahl *et al.*, 2013). DPPA3 is also abundant in bovine oocytes and preimplantation embryos and its role in preventing early DNA demethylation has been recapitulated in bovine embryos, thus it could be hypothesised that a similar process occurs in early human embryos (Bakhtari and Ross, 2014). In larger animal models, DPPA3 therefore appears to be a marker of germ cells at a slightly later stage in development than LIN28 and NANOG.

1.2.2.6 IFITM3

IFITM3, also known as Fragilis, is a cell surface protein which is evolutionarily conserved, with homologues detected in the human and bovine genomes (Saitou *et al.*, 2002, Lange *et al.*, 2003). It was first identified in mouse embryos, with strong, specific expression seen in the area where PGCs specification occurs (Saitou *et al.*, 2002). There are conflicting data regarding its role in migratory PGCs, with some research suggesting it is present (Tanaka and Matsui, 2002) and others reporting that it is markedly downregulated (Saitou *et al.*, 2002), with knock-out studies demonstrating that Ifitm3 is not necessary for normal germ cell development, including migration (Lange *et al.*, 2008). A possible role for Ifitm3 in PGC specification has been demonstrated, however, by examining the relationship between Bmp4 and Ifitm3 expression, with Bmp4-null mutants exhibiting no Ifitm3 expression (Saitou *et al.*, 2002). With regards the timing of its expression, Ifitm3 appears to precede that of Dppa3, with the latter only expressed in early PGCs with high levels of Ifitm3 (Saitou *et al.*, 2002).

Human data are lacking, although bovine studies have demonstrated that IFITM3 expression is confined to the epiblast of the blastocyst, from which PGCs are specified (Smith *et al.*, 2007). Given its conservation across species, however, it is likely that IFITM3 is a reliable marker of germ cells in humans and cows.

1.2.2.7 C-KIT

C-KIT is a cell surface tyrosine kinase receptor whose ligand, SCF (also known as Kit ligand (KL)), causes dimerisation of C-KIT and downstream phosphorylation of intracellular pathways (Saiti and Lacham-Kaplan, 2007). Its role in murine germ cell development is long established, with expression detected from the time of PGC specification to the point of meiotic entry, then disappearing until oocytes enter the diplotene stage (Manova and Bachvarova, 1991). Mutations in the *c-kit* gene do not affect PGC specification, but does have a detrimental effect on PGC proliferation, indicating its mitogenic role (Besmer *et al.*, 1993). In the post-natal ovary, oocytes express c-kit, whilst surrounding granulosa cells express SCF; mutations affecting SCF expression lead to follicular arrest at the primary stage, suggesting that these molecules are involved in oocyte growth (Besmer *et al.*, 1993).

Human C-KIT expression has been demonstrated in oogonia and post-natal oocytes at all stages of development (Horie *et al.*, 1993, Robinson *et al.*, 2001, Stoop *et al.*, 2005). The interspecies variation in expression seen in POU5F1 between mouse and human is again observed, with human C-KIT expression persisting throughout fetal development (Stoop *et al.*, 2005). This difference in both POU5F1 and C-KIT expression may be explained by the asynchronous entry of human PGCs into meiosis: this may result in mitotic PGCs continuing to express C-KIT whilst those undergoing meiosis cease to produce it, thereby there is no hiatus in apparent expression as seen in the mouse. In contrast to POU5F1 expression, however, C-KIT expression is maintained in oocytes after POU5F1 expression has declined (Stoop *et al.*, 2005). C-KIT also has roles in melanogenesis and haematopoiesis (Besmer *et al.*, 1993), therefore, as with PRDM1, cannot be regarded as a germ cell-specific marker.

1.2.2.8 DDX4

DDX4 (or VASA) is an intracytoplasmic, ATP-dependent RNA helicase which is reported to be exclusively expressed by germ cells: expression in pluripotent cells and non-gonadal tissue has not been demonstrated (Castrillon *et al.*, 2000, Toyooka *et al.*, 2000). In both mice and humans, it is first detected in PGCs colonising the genital ridges and continues to be expressed throughout the rest of the germ cell

developmental pathway, with expression persisting in post-meiotic oocytes (Castrillon *et al.*, 2000, Toyooka *et al.*, 2000, Stoop *et al.*, 2005). In humans, its expression increases as the oocyte matures and, as with C-KIT, its initial co-localisation with POU5F1 ceases once folliculogenesis commences (Castrillon *et al.*, 2000, Stoop *et al.*, 2005). Bovine studies have not been as detailed, but demonstrate that DDX4 expression is constrained to germ cells in the fetus (Bartholomew and Parks, 2007) and can be detected up to the antral follicle stage in adult ovaries (Pennetier *et al.*, 2004). *In vitro* co-culture studies have suggested that its expression is "switched on" by cell-cell interactions with somatic cells within the genital ridge (Toyooka *et al.*, 2000) and its role in germ cell development appears to be relatively conserved across species, including *C. elegans*, *Drosophila* and mice, with knock-out studies demonstrating various germline deficiencies (Ewen-Campen *et al.*, 2010). DDX4 is thus a well-established germ cell-specific marker across the phylogenetic spectrum (Ewen-Campen *et al.*, 2010).

1.2.3 Female GSCs in Non-Mammalian Species

The role of fGSCs is well-established in several animals (reviewed in Dunlop *et al.*, 2014), in particular "lower" invertebrates such as *C. elegans* and *Drosophila* (Fig. 1.5). In *C. elegans*, a GSC population is formed in the larvae and, in the adult, these cells maintain a stable population of germ cells and are constantly producing gametes (Kimble, 2011). Around 35-70 GSCs reside in a germ cell niche at the distal end of the gonad, called the distal tip cell (DTC), which maintains their self-renewal properties (Kimble and White, 1981); however, as the GSCs extend outside the niche, they begin to mature and enter meiosis, with maturation occurring in the distal-to-proximal direction (Kimble, 2011). The Notch signaling pathway is a key regulator in this process: it both stimulates GSC mitosis and is essential in repressing meiotic entry, thereby maintaining the GSC population (Kimble, 2011).

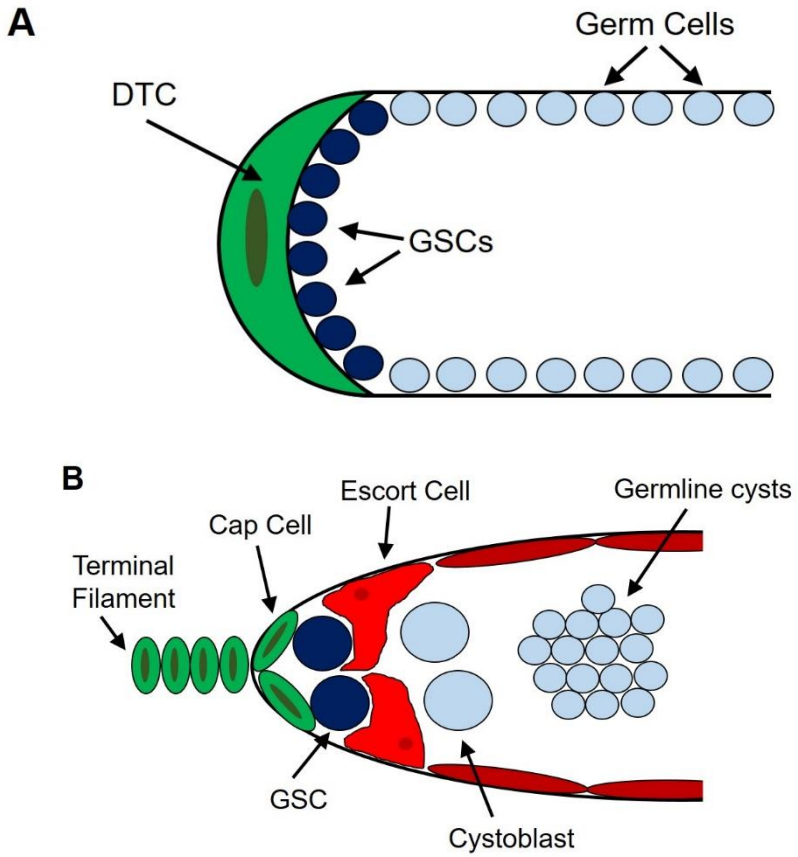


Figure 1.5. The germ cell niches of (A) *C. elegans* and (B) *Drosophila*. Proximity of the DTC maintains *C. elegans* GSCs in their pluripotent state and thus they mature in a distal-to-proximal direction. The germarium of *Drosophila* contain escort cells which prevent GSCs from being in contact with each other. GSCs divide to become cystoblasts which generate 16 germline cysts. DTC = distal tip cell, GSC = germline stem cell. Adapted from Chen *et al.*, 2013.

In *Drosophila*, ovaries are made up of 16 to 18 tubes, called ovarioles, and a germ cell niche, containing approximately two GSCs, is found at the tip of each ovariole, termed the germarium (Xie and Spradling, 2000). The niche comprises three different somatic cell types: terminal filament cells at the anterior end of the ovariole, caps cells which are associated with the GSCs and escort cells which prevent the GSCs from touching each other (Spradling *et al.*, 2011). GSCs mitose asymmetrically, forming a GSC and a cystoblast cell, with the former maintaining the GSC population by remaining beside

the cap cells, and the latter continuing to divide until 16 germline cysts have been produced. Only one of these cells will become an oocyte, while the remainder become supportive or 'nurse' cells (Spradling *et al.*, 2011). The regulatory pathways involved in maintaining the *Drosophila* GSC population and promoting differentiation are complex but, in brief, the terminal filament cells activate the JAK-STAT signaling pathway within the cap cells which, in turn, induces the production of BMP ligands. In response, the GSCs repress the expression of an inducer of differentiation, *bag-of-marbles* (*bam*) and maintain high levels of the translational repressor NANOS, thereby sustaining GSC function and preventing differentiation. Once the GSCs have left the niche, however, they are no longer exposed to BMP ligands, therefore *bam* is upregulated and daughter cells can differentiate into germ cells (Spradling *et al.*, 2011).

Female GSCs have also been described in non-mammalian vertebrates, including some species of fish. Teleost fish such as zebrafish (*Danio rerio*) (Draper *et al.*, 2007) and the medaka (*Oryzias latipes*) (Nakamura *et al.*, 2010) have been reported to undergo post-natal *neo*-oogenesis. It has been postulated that the germ cell niche in zebrafish is situated in the germinal zone, a discrete area on the ovarian surface (Beer and Draper, 2013). Furthermore, as in *Drosophila*, the Nanos protein appears to be essential for ongoing oogenesis in the adult ovary of the zebrafish, with the *nanos1, nanos2* and *nanos3* genes all implicated in maintaining the fGSC population (Draper *et al.*, 2007, Beer and Draper, 2013). In the medaka, the germ cell niche is termed the germinal cradle and is located in the ovarian cords (Nakamura *et al.*, 2010). Once again, Nanos has been found to be important, with mitotically-active medaka fGSCs expressing *nanos2* (Nakamura *et al.*, 2010). The existence of specialised niches across phyla with some similarities in regulatory pathways is indicative of evolutionary conservation within the animal kingdom.

1.2.4 The Evidence for Female GSCs in Mammals

In such fertile species as flies and fish, where oocytes have to be produced in large quantities over the animal's lifespan, as with sperm in mammals, it seems logical that *neo*-oogenesis is continuous in adulthood. However, evidence of fGSCs has also been reported in some mammalian species which are much less fecund than fish or flies.

Indeed, under certain conditions, oocytes and even offspring have been created from such cells (Fig. 1.6).

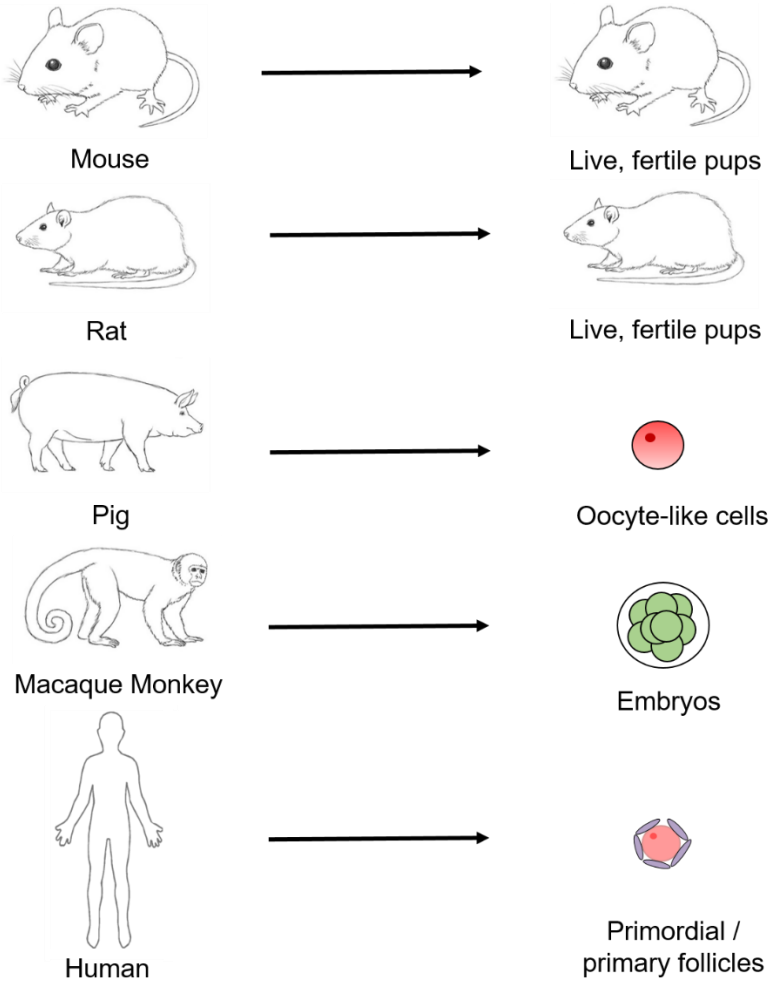


Figure 1.6. Putative female germline stem cells have been isolated in five species thus far, with live mice and rat pups, porcine oocyte-like cells, macaque monkey embryos and human immature follicles generated from them under specific conditions (Zou *et al.*, 2009, White *et al.*, 2012, Wolff *et al.*, 2013, Bui *et al.*, 2014, Zhou *et al.*, 2014, Wolff *et al.*, 2014, Xiong *et al.*, 2015, Lu *et al.*, 2016, Wolff, 2016).

1.2.4.1 Mice

Jonathan Tilly's group were investigating follicular atresia in a mouse model when the authors reached an unintended, and surprising, conclusion: that their findings could

only be explained by *neo*-oogenesis (Johnson *et al.*, 2004). The authors performed follicle counts over the reproductive lifespan of the C57BL/6 mouse and discovered that, given the rate of follicular degeneration, the ovary should be exhausted of oocytes much earlier than is observed *in vivo*. To ensure that they were not counting accumulating atretic follicles, they chemically synchronised the atresia of primordial and primary follicles and found that unhealthy oocytes were cleared within 3 days. They confirmed their findings using two other mouse strains: CD1 and AKR/J. By comparing their numerical findings to those of a previously reported model (Faddy *et al.*, 1987), they surmised that approximately 77 new follicles would be required daily in order to sustain the mouse follicular pool until reproductive senescence. Further investigation revealed the existence of ovoid cells in the ovarian surface epithelium (OSE) which dual-stained for Ddx4 and 5-bromodeoxyuridine (BrdU), a marker of proliferation (Johnson *et al.*, 2004). These cells were not contained within a follicular structure and were found to be in differing stages of mitosis. Discrete cells expressing Sycp3 (a marker of meiotic entry) in the OSE were also reported, implying that a population of mitotically active germ cells capable of entering meiosis and thus forming new oocytes, was present in the adult mouse ovary. The authors were further convinced of this hypothesis when wild-type ovarian fragments transplanted onto ubiquitously green fluorescent protein (GFP)-expressing mouse ovaries contained GFP-positive oocytes surrounded by non-fluorescent wild-type granulosa cells in the grafted tissue after 3-4 weeks (Johnson *et al.*, 2004). The ability of these oocytes to be fertilised was not assessed, therefore their functional capabilities were unknown.

Follicular quantification has subsequently endorsed Johnson *et al*.'s follicular kinetics findings (Johnson *et al.*, 2004), thus providing further indirect evidence of post-natal *neo*-oogenesis in mice. Using stereological methods, it has been demonstrated that in adult C57BL/6 mice the average number of primordial follicles remained stable from post-natal days 7 to 100, with a decline in numbers only observed following this time period (Kerr *et al.*, 2006). One possible explanation for this apparent follicle stability is that new follicles are being formed in the adult, thus, although this study did not identify any fGSC population, it does support the concept of *neo*-oogenesis.

The concept has also been indirectly supported by analysis using lineage tracing. A study in mice examined how many mitotic divisions an oocyte had undergone since it

was formed, also known as oocyte "depth", by analysing microsatellite mutations retrospectively (Reizel *et al.*, 2012). Microsatellites are nucleotide repeats in non-coding areas of DNA (therefore mutations within them do not have a phenotypic effect on the animal) and mutation information can be used to indicate the oocyte "depth". It was discovered that oocyte "depth" increases with age, i.e. the oocytes of older mice had undergone more mitotic divisions than those of younger mice. Two principal hypotheses could help explain this finding: (1) the "production line hypothesis" which states that oocytes recommence meiosis in the order in which they were formed in the fetal gonad (Henderson and Edwards, 1968), so that oocytes in older mice will have undergone more proliferation pre-natally than younger oocytes, or (2) the post-natal *neo*-oogenesis hypothesis, as oocytes with increased "depth" may have been formed by continually dividing OSCs in the adult ovary. Interestingly, the "depth" was further increased in mice who had one ovary removed (Reizel *et al.*, 2012), which again could be explained by post-natal *neo*-oogenesis within the remaining ovary.

Further studies by Tilly's group focussed on the location of the putative fGSCs, as the scarcity of the population within the ovary implied that there may be an extra-ovarian source (Johnson *et al.*, 2005a). Given the common embryonic origin of haematopoietic stem cells and PGCs in the proximal epiblast, the authors directed their studies towards bone marrow (BM) and reported that BM expressed several germ cell markers at the mRNA level, including *Ddx4, Ifitm3* and *Dazl*. In addition, mice that had been sterilised with cyclophosphamide and busulfan chemotherapy and subsequently received a BM transplant (BMT) from a healthy mouse, had their ovarian function restored, with follicles of every developmental stage seen by histological analysis (Johnson *et al.*, 2004, Lee *et al.*, 2007). This was in direct contrast to chemotherapy-sterilised mice who did not receive a BMT, whose ovaries contained very few follicles (Johnson *et al.*, 2005a). This phenomenon did not seem to be restricted to BM: a peripheral blood stem cell transplant (PBSCT) from transgenic mice in which a *Pou5f1* promoter drives GFP expression, appeared to also replenish chemotherapy-sterilised ovaries, with GFP-positive oocytes seen in primordial follicles less than 2 days after PBSCT (Johnson *et al.*, 2005a). The developmental competence of such oocytes was not tested, however.

Transgenic mice which expressed GFP under the control of *Pou5f1* were also used to demonstrate the existence of putative fGSCs in separate research (Zhang *et al.*, 2008). By tracking GFP, Zhang *et al*. detected oval aggregates in the ovarian cortex which were not contained in follicular structures. The aggregates were isolated from dissociated ovary by visualisation under a fluorescent microscope and demonstrated co-localisation of Pou5f1, Ddx4 and c-kit by immunofluorescence. Furthermore, the aggregates expressed meiotic entry genes (*SYCP3* and *disrupted meiotic cDNA 1*; *DMC1*) at the mRNA level, but not *growth differentiation factor-9* (*GDF-9*), which is a marker of mature oocytes. The authors therefore postulated that these aggregates contained putative fGSCs of which a subpopulation was undergoing folliculogenesis, with oogonia transitioning to oocytes (Zhang *et al.*, 2008).

Yet, none of these studies had isolated a pure population of fGSCs and assessed their ability to undergo both oogenesis and fertilisation. It wasn't until 2009 that there was a breakthrough in the field, when Zou *et al*. reported the isolation of fGSCs from neonatal and adult mouse ovaries which produced developmentally competent oocytes and, upon fertilisation, live pups (Zou *et al.*, 2009). The authors confirmed the existence of cells which stained for both Ddx4 and BrdU in the OSE and went on to use Ddx4 to select putative fGSCs from disaggregated neonatal and adult ovarian tissue by immunomagnetic methods. Characterisation of the isolated cells revealed a euploid karyotype, maternal imprinting and a molecular signature expected of a GSC, with the expression of both pluripotency (e.g. *Pou5f1*) and germline (e.g. *Ddx4*, *Ifitm3*) markers. The stem cell quality of the cells was confirmed by the presence of high telomerase activity and alkaline phosphatase (ALP) staining. Transfection of both the neonatal and adult putative fGSCs with GFP-containing retrovirus and transplantation into the ovaries of chemoablated mice revealed GFP-positive oocytes within the host ovaries within 2 months. Furthermore, mating of recipient mice with wild-type males resulted in GFP-positive offspring (27% of offspring in neonatal fGSC-transplanted mice and 28% in adult fGSC-transplanted mice). These offspring were themselves fertile and produced GFP-positive offspring in the F2 generation. Importantly, injection of the cells subcutaneously into nude mice did not cause teratomas, indicating that the cells were restricted to germ cell differentiation (Zou *et al.*, 2009). Consequently, it has been postulated that fGSCs are established at a point in the PGC

differentiation pathway after migration and before germ cell nest formation (Abban and Johnson, 2009), as post-migratory mouse PGCs can become embryonic germ cell lines that form teratomas (Shim *et al.*, 2008).

The same group has subsequently demonstrated that putative fGSCs can also be isolated using an antibody against a different germ cell marker, Ifitm3, with greater efficiency than that found using Ddx4 (Zou *et al.*, 2011). Ddx4- and Ifitm3-isolated cells had the same characteristics and were considered to be the same cell type.

The isolation and culture of putative mouse fGSCs has since been reported by a handful of other groups (Pacchiarotti *et al.*, 2010, Hu *et al.*, 2012, White *et al.*, 2012, Hernandez *et al.*, 2015, Xiong *et al.*, 2015, Lu *et al.*, 2016) using varying methodologies. Pacchiarotti *et al*. used transgenic mice in which GFP expression was driven by a *Pou5f1* promoter and isolated putative Pou5f1-expressing fGSCs by detecting GFP during fluorescence-activated cell sorting (FACS) (Pacchiarotti *et al.*, 2010). These cells expressed other germline markers (Ddx4 and c-kit) at the protein level and did not form teratomas. The cells appeared to spontaneously form larger oocyte-like cells (OLCs) during *in vitro* culture and also aggregated with neonatal granulosa cells when cultured in hanging drops of culture medium to form structures reminiscent of primordial follicles; however, subsequent development and fertilisation capabilities were not tested. Of note, the authors of this paper did not find that the GFP-Pou5F1-positive cells, that they supposed were putative fGSCs, expressed the proliferative marker proliferating cell nuclear antigen (PCNA), *in situ* within the OSE. They therefore hypothesised that these cells did not have a physiological role in the normal post-natal ovary and, as such, remained quiescent unless activated by specific environmental circumstances (Pacchiarotti *et al.*, 2010).

Hu *et al*. did not perform any selection process in order to isolate putative fGSCs in adult mice ovary: they simply dissociated the ovaries, plated them onto a feeder layer of mouse embryonic feeder (MEF) cells and analysed the colonies that established in culture (Hu *et al.*, 2012). The colonies stained for ALP and expressed pluripotency markers (*Pou5f1*, *Nanog*) at the mRNA level and both pluripotency (Pou5f1, Nanog) and germline (Ddx4) markers at the protein level using immunofluorescence, thereby convincing the authors that the cells were putative fGSCs. Additionally, the authors

reported that, in contrast to previous assertions, these cells were located around the follicles in the ovarian cortex, rather than the OSE. When the cells were suspended in culture, they formed embryoid bodies (EB) and, in the presence of porcine follicular fluid (PFF), differentiated into larger OLCs which had upregulated expression of oocyte markers (*Figla*, *zona pellucida 1*, *2* and *3* (*ZP1*, *2* and *3*). However, the EBs could also be induced to form neurones, cardiomyocytes and pancreatic cells under specific *in vitro* culture conditions, suggesting the cells could be reprogrammed to attain pluripotency (Hu *et al.*, 2012).

Both White *et al*. and Hernandez *et al*. used the same isolation process, utilising FACS to select for Ddx4-positive cells from dissociated adult mouse ovary (White *et al.*, 2012, Hernandez *et al.*, 2015). In their paper, White *et al*. coined the term oogonial stem cells (OSCs), which can be regarded as interchangeable with fGSC (White *et al.*, 2012). Given that the data within this thesis was derived using the same isolation methodology as White *et al*. (White *et al.*, 2012), and in the interest of consistency, the term OSC will be used for the remainder of this thesis when referencing mammalian fGSCs. White *et al*. reported the existence of a scarce population of putative mouse OSCs, comprising 1.5% ± 0.2% of the mixed cell population used in the experiments (White *et al.*, 2012). As only the cortex was utilised, this translated to a prevalence of 0.014% ± 0.002% within the entire ovary. Freshly isolated cells expressed mRNA of germline markers, including *Ddx4*, *Ifitm3*, *Prdm1* and *Dazl*, and this expression was retained after long-term propagation in *in vitro* culture. The cells did not produce teratomas. As seen previously (Pacchiarotti *et al.*, 2010), OLCs which expressed oocyte markers (including *Zp1-3*, *Gdf-9* and newborn ovary homeobox (*Nobox)*) at the mRNA level were generated spontaneously during *in vitro* culture. Furthermore, ploidy analysis revealed that some cultured cells attained haploid status indicating that they may have undergone meiosis. The oogeneic potential of the putative OSCs was further tested by transducing the cells to express GFP using a GFP-containing retrovirus and injecting the cells into the ovaries of adult mice. GFP-positive oocytes were detected within growing follicles after 5-6 months and, after ovulation hyperstimulation, cumulus-oocyte complexes (COC) were collected from the oviducts, of which some COCs contained a GFP-positive oocyte associated with wild-type granulosa cells. These GFP-positive oocytes could be fertilised and reached

the hatching blastocyst stage, although the ability of these blastocysts to create liveborn pups was not verified. Follow-up studies revealed that, as well as retaining germline marker expression, mouse OSCs gain pluripotency marker expression (e.g. *Pou5f1*, *Nanog*) during *in vitro* culture, with no pluripotency markers detected in freshly isolated cells (Imudia *et al.*, 2013). This pattern is analogous to SSCs, which attain multipotency in culture (Guan *et al.*, 2007). The molecular similarities of OSCs and SSCs has been confirmed by Wu's group, who demonstrated that the two cell types express 853 of the same genes on microarray analysis, equivalent to over half of the highly expressed genes detected in the two cell types (Xie *et al.*, 2014).

Hernandez *et al*.'s findings supported the rarity of this population, with 1.9 – 3.7% of ovarian cells being Ddx4-positive (Hernandez *et al.*, 2015). Although these cells expressed the germline markers *Ifitm3* and *Prdm1*, paradoxically the authors were unable to detect *Ddx4* expression, including after *in vitro* expansion, which contradicts the previous findings detailed above. No functional testing of these putative OSCs was performed.

The use of immunomagnetic sorting for Ifitm3 has been reproduced by another group recently (Xiong *et al.*, 2015, Lu *et al.*, 2016). The isolated cells expressed germline markers (Ifitm3, Ddx4, Prdm1), had a normal karyotype and injection of GFP-expressing OSCs into the ovaries of sterilised mice generated GFP-positive F1 offspring (Lu *et al.*, 2016). The cells could also be isolated from mice that had undergone chemotherapy, and after expansion and GFP-transfection *in vitro*, injection into sterilised mice generated GFP-positive offspring (Xiong *et al.*, 2015).

Research has shown that the prevalence of putative OSCs decreases with age, with Pacchiarotti *et al*. reporting a decrease in population size from 1-2% of the neonatal ovary, to 0.05% of the adult ovary (Pacchiarotti *et al.*, 2010). However, putative OSCs have also been reported in aged mouse ovaries (Niikura *et al.*, 2009). Immuno-localisation demonstrated the existence of small clusters of Stra8-producing cells in the ovarian surface epithelium (OSE) implying that pre-meiotic germ cells are present, but are perhaps not able to progress through meiosis to become functional oocytes. However, upon introduction of these cells into a younger ovarian environment, primordial follicle structures were produced, which expressed the oocyte marker,

Nobox (Niikura *et al.*, 2009). This infers that the conditions of the niche surrounding such stem cells are just as important as the cells themselves in allowing their full potential to be achieved.

Finally, with regards mouse research, very recently published research has provided some evidence for a physiological role for OSCs: lineage tracing of transgenic adult mice that expressed yellow fluorescent protein (YFP) in response to a Pou5f1 promoter revealed non-follicular Pou5f1-expressing "small germ cells" which also co-expressed varying levels of Ddx4, Dazl, Sycp3 and Stra8 (Guo *et al.* 2016). In addition, some of these cells stained for BrdU, indicating that a population of mitotically-active cells are indeed present in the adult mouse ovary, which possibly sequentially acquire the expression of germline and meiotic entry markers and may thus contribute to the follicular pool (Guo *et al.*, 2016). The re-expression of Pou5f1 in oocytes at later stages of development precludes this model from providing more robust evidence as to the subsequent developmental potential of these labelled cells.

1.2.4.2 Rats

Using the same Ifitm3-based immunomagnetic sorting technique as used in mice (Zou *et al.*, 2011), putative OSCs have been isolated from adult rat ovaries (Zhou *et al.*, 2014). Zhou *et al*. demonstrated cells in the OSE which dual-stained for Ifitm3 and BrdU, suggesting these were putative OSCs (Zhou *et al.*, 2014). Isolated cells had a euploid karyotype, stained for ALP and had high telomerase activity, indicative of stem cells. They demonstrated mRNA expression of pluripotency (*Pou5f1*) and germline markers (*Ifitm3*, *Ddx4*, *Dazl*) and spontaneously differentiated in *in vitro* culture into OLCs which expressed Zp3. As seen in Zou *et al*.'s mouse study (Zou *et al.*, 2009), GFP-expressing rat OSCs produced GFP-positive oocytes when injected into recipient rats and, furthermore, produced live GFP-positive offspring after mating, thus proving their developmental competency.

1.2.4.3 Bats

Another mammal where potential OSCs and a germ cell niche have been identified is the Phyllostomidae family of bats. Interestingly, the reproductive system of these bats is comparable to primates, with anatomical and menstrual cycle similarities: *Glossophaga soricina* bats are monovular and polyoestrous, with 22-26 day menstrual cycles, a luteal phase and menstruation (Rasweiler, 1972). *G. soricina* bats, along with *Artibeus jamaicensis* and *Sturnira lilium* bats, have polarised ovaries with follicles and corpora lutea located in the medulla and non-growing primordial follicles situated in the cortical region (Antonio-Rubio *et al.*, 2013). Immunohistochemistry revealed a population of cells with morphological similarities to PGCs, which the authors termed adult cortical germ cells (ACGCs), and further investigation with immunofluorescence indicated that these cells simultaneously expressed markers of proliferation (phosphorylated-histone 3; H3ph), pluripotency (POU5F1 and DPPA3) and germ cells (DDX4, IFITM3 and C-KIT), leading the authors to conclude that ACGCs may contribute to *neo*-oogenesis in the phyllostomid bat (Antonio-Rubio *et al.*, 2013). However, although some of these ACGCs appeared to be in primordial follicle-like structures, their ability to complete folliculogenesis and definitively replenish the ovarian reserve has not been established.

1.2.4.4 Pigs

Putative OSCs have been reported in the ovaries of both juvenile (Bai *et al.*, 2013) and adult (Bui *et al.*, 2014) pigs. Studies in juvenile (4 – 6 months old) porcine ovaries appeared to demonstrate that putative OSCs, as characterised by the expression of POU5F1 and DDX4, were present in the theca layers of the ovaries, rather than the OSE (Bai *et al.*, 2013). Cells were isolated by performing cell scrapings between the OSE and the medullary region, were cultured *in vitro* and demonstrated pluripotency (e.g. POU5F1) and germ cell (e.g. DDX4, C-KIT) expression by immunofluorescence. Their reproductive capacity was not investigated; however, the cells could differentiate in *in vitro* culture into many different cell types and formed teratomas when transplanted into mice (Bai *et al.*, 2013). This indicates their pluripotent nature, which

contradicts the findings of apparent unipotency in mice (Zou *et al.*, 2009, Pacchiarotti *et al.*, 2010, White *et al.*, 2012).

Adult porcine putative OSCs were initially isolated by a similar method to Hu *et al*.'s methodology in mice (Hu *et al.*, 2012), whereby ovaries were dissociated and grown on a feeder layer (Bui *et al.*, 2014). Flow cytometry of putative OSCs after 1 week of *in vitro* expansion, perhaps unsurprisingly, revealed a heterogeneous population, differing in size (a quarter of cells were 5-7µm and the remainder were 10-12µm) and gene expression, with a small minority of cells expressing DDX4, IFITM3 and C-KIT. PCR demonstrated that the cells expressed both stem cell markers (including *POU5F1*, *NANOG*) and germline markers (including *DDX4*, *IFITM3*, *C-KIT*, *PRDM1*) at the mRNA level, both after 1 week and 4 weeks of culture. The cells could be propagated in culture long-term, with no loss of proliferative capacity, stained positively for ALP, had a normal karyotype and did not form teratomas in nude mice. OLCs, as identified by morphology, were spontaneously formed *in vitro* and when cells were cultured in a differentiation medium containing PFF, the OLCs that formed expressed oocyte markers including *ZP*, *SYCP3* and *GDF-9*. The group developed their research further and sorted cells immunomagnetically for stage specific embryonic antigen 4 (SSEA4), a stem cell marker (Bui *et al.*, 2014, Henderson *et al.*, 2002). These cells similarly appeared to differentiate *in vitro* into OLCs and when transfected GFP-expressing cells were injected into porcine ovarian cortex and xenografted into nude mice, cells co-expressing GFP and oocyte markers (e.g. GDF-9) could be detected. Folliculogenesis and fertilisation competency was not assessed.

1.2.4.5 Cows

To date, no direct evidence exists that OSCs exist in post-natal bovine ovaries with no isolation of such cells reported. However, similar to the findings described in the mouse (Johnson *et al.*, 2004), a study examining the senescence of bovine ovaries reported high levels of follicular atresia during the prime reproductive period of the cow, despite both the non-growing (primordial) and growing follicle population remaining relatively stable (Erickson, 1966). Although the underlying reason for this was not further elucidated, one such reason could be that the follicular pool is

undergoing replenishment during that period, with reproductive senescence occurring once ageing of the OSCs and/or the germ cell niche prevents further *neo*-oogenesis.

1.2.4.6 Prosimian Primates

Several prosimian primates have been found to have mitotically-active germ cells in the adult ovary, including the brown greater galago (*Galago crassicaudatus*), the Demidoff's bushbaby (*G. demidoffi*) and the potto (*Perodicticus potto*) (Ioannou, 1967). These cells were found in germ cell nests located in the adult ovarian cortex and tritiated thymidine studies demonstrated DNA synthesis activity (Ioannou, 1967). Similar studies on the Senegal galago (*G. senegalensis*) concluded that, by examining staining intensity and meiotic stage, some labelled oogonia had undergone at least one mitotic division and some had entered meiosis in the 10 days between labelling and ovariectomy (Butler and Juma, 1970). In addition, the loris family are prosimian primates related to the lemur, of which two members have been described as having post-natal proliferating germ cells: one member of each of the loris subcategories, a slow loris (*Nycticebus coucang*) (Duke, 1967) and a slender loris (*Loris tardigradus lydekkerianus*) (Ioannou, 1967, David *et al.*, 1974). These studies also demonstrated uptake of tritiated thymidine by collections of cells within the ovarian cortex; however, the ability of these cells to undergo post-natal oogenesis and folliculogenesis has not been proven.

1.2.4.7 Non-human primates

Indirect evidence that monkeys may undergo post-natal *neo*-oogenesis was provided in the middle of the 20th century, when investigations into follicular atresia in the rhesus macaque monkey (*Macaca mulatta;* an Old World monkey) were published (Vermande-Van Eck, 1956). Just as Johnson *et al*. described in their mouse studies (Johnson *et al.*, 2004), Vermande-Van Eck determined that the rate of atresia was such that the ovarian reserve should be exhausted within 2 years (Vermande-Van Eck, 1956). In reality, rhesus macaque monkeys do not go through the menopause until approximately 25 years old (Walker and Herndon, 2008). From her observations, Vermande-Van Eck calculated that the average lifespan of a rhesus macaque oocyte

was 6 months, with a maximum lifespan of approximately 2 years, thus she concluded that to allow for a reproductive lifespan of more than two decades, oogenesis must continue in adulthood (Vermande-Van Eck, 1956).

This line of evidence has been supported by the reported isolation of OSCs from the same species of monkey (Hernandez *et al.*, 2015). Using the published FACS protocol utilising DDX4 for cell selection (White *et al.*, 2012, Woods and Tilly, 2013), a DDX4-positive population of cells comprising 2.5 – 50.6% of ovarian cells was isolated from 17 different monkeys (Hernandez *et al.*, 2015). After *in vitro* expansion, these putative OSCs were fluorescently tagged with GFP using lentiviral transduction before autologous injection was performed. Four months later, the monkey underwent ovarian hyperstimulation and 5 oocytes were aspirated (including 4 MII oocytes and 1 immature oocyte), with one MII oocyte expressing GFP as confirmed by fluorescence microscopy and polymerase chain reaction (PCR) (Wolff *et al.*, 2013, Wolff *et al.*, 2014). This was the first demonstration of an OSC-derived mature oocyte in primates, and fertilisation and early embryo development (but not pregnancy) has subsequently been reported (Wolff, 2016).

Recently, the possibility that post-natal *neo*-oogenesis may also occur in New World monkeys has been raised (Fereydouni *et al.*, 2014, Fereydouni *et al.*, 2016). Research in neonatal common marmoset monkeys (*Callithrix jacchus*) has demonstrated that they possess primitive ovaries containing many oogonia which retain a pluripotent molecular mRNA signature (*POU5F1* and *LIN28*) despite being post-natal (Fereydouni *et al.*, 2014). Immunohistochemistry demonstrated pre-meiotic, POU5F1- and LIN28-positive germ cells as well as more mature, POU5F1- and LIN28-negative germ cells and by the time the monkey was 1 year old, no POU5F1 or LIN28 expression was detectable. Cells from dissociated neonatal ovaries could be cultured long-term on feeder layers, did not produce teratomas and spontaneously produced OLCs which expressed germ cell markers (including *DDX4* and *DPPA3*) and a marker of meiotic entry (*SYCP3*) (Fereydouni *et al.*, 2016). However, in contrast to previous studies into OSCs and the findings in whole neonatal ovaries, these cultured cells did not express markers of OSCs until later passages (e.g. *LIN28*, *DPPA3*, *PRDM1*, *DDX4*) or, in some cases, did not express them at all (e.g. *POU5F1*, *NANOG*), suggesting that they are not OSCs, as defined by previous groups. The

authors hypothesised that this incongruity may be due to the fact that the cultured cells contained OSE cells with stem cell capabilities and the pluripotency marker-expressing oogonia did not survive in the culture environment (Fereydouni *et al.*, 2016). Investigation into the *in vitro* oogeneic potential of adult marmoset ovarian cells was not performed.

1.2.4.8 Humans

When the research contained within this thesis was commenced, there had only been one report of OSCs being isolated in humans (White *et al.*, 2012). Since then, one other group has reported that they have also been successful using the same protocol as White *et al*., although with differing molecular findings (Hernandez *et al.*, 2015). Utilising the same DDX4-selecting FACS protocol as in their mouse studies, White *et al*. demonstrated that a rare population of DDX4-positive cells could be isolated from human ovarian cortex, comprising 1.7% ± 0.6% of the ovarian cortex (White *et al.*, 2012). Of note, the group have subsequently reported that cells have been found in women in their 50s, indicating they are not solely a phenomenon of young women (unpublished data cited within Woods and Tilly, 2012). The cells expressed germ cell markers (including *DDX4, IFITM3, C-KIT, PRDM1*), spontaneously generated larger OLCs during *in vitro* culture which expressed the oocyte markers Y-box binding protein 2 (YBX2) and LIM homeobox protein 8 (LHX8) at the protein level and some cells exhibited haploid status (White *et al.*, 2012). The putative OSCs were reported to undergo oogenesis both *in vitro* and *in vivo* with detection of the differentiated OSCs performed by transfecting them with a GFP-containing retrovirus beforehand. *In vitro*, aggregates of putative OSCs and dissociated ovarian cortex produced large GFP-positive cells surrounded by GFP-negative cells in follicle-like structures within 3 days of culture. Xenografting of GFP-OSC injected human ovarian cortex into immunodeficient mice revealed GFP-positive oocytes surrounded by non-GFP cells in primordial or primary follicles within 7 days of transplantation (White *et al.*, 2012). More mature follicles were not generated and both ethical and legal reasons currently prevent assessment of the fertilisation capabilities of any mature oocytes.

Hernandez *et al*. used an identical isolation protocol, but in contrast to the above study the size of the DDX4-positive population was larger and more variable, comprising 4.5 – 24% of ovarian cells (Hernandez *et al.*, 2015). Furthermore, and consistent with the group's mouse and rhesus macaque monkey findings in the same paper, although the cells expressed germ cell markers (*PRDM1, IFITM3, DPPA3*), they did not appear to express DDX4, either using PCR or mass spectrometry, despite this being the protein the cells were selected for. The cells appeared to generate OLCs spontaneously during *in vitro* culture, but no further oogeneic assessments were performed (Hernandez *et al.*, 2015).

In summary, there is a growing body of evidence for the presence of OSCs and the process of post-natal *neo*-oogenesis in mammals, although in contrast to non-mammalian species, a germ cell niche has not been described. However, the concept remains controversial, with opponents being sceptical of the findings and producing data in support of the fixed ovarian reserve dogma.

1.2.5 The Arguments against *Neo*-oogenesis and OSCs

Critics of the *neo*-oogenesis hypothesis have three principal issues with the findings discussed above:

1) The data produced by OSC proponents are open to alternative explanations (Gosden, 2004, Byskov *et al.*, 2005, Telfer *et al.*, 2005, Notarianni, 2011, Oatley and Hunt, 2012).
2) There are questions regarding the validity of the use of DDX4 in isolation methodologies (Telfer and Albertini, 2012, Albertini and Gleicher, 2015, Hernandez *et al.*, 2015).
3) Reproducibility of results has proved problematic (Eggan *et al.*, 2006, Zhang *et al.*, 2015).

This section will expand on these points and discuss rebuttals made by supporters of the concept of an adult mammalian OSC.

1.2.5.1 Alternative Explanations for the Data

This was the first matter of contention following the publication of Johnson *et al*.'s 2004 and 2005 studies (Johnson *et al.*, 2004, Gosden, 2004, Byskov *et al.*, 2005, Johnson *et al.*, 2005a, Telfer *et al.*, 2005). With regards the follicular atresia observations in the 2004 paper, it was highlighted that classification of follicle health by morphology alone is subjective and fixing of tissue may result in an overestimate of follicular atresia (Gosden, 2004, Greenfeld and Flaws, 2004, Byskov *et al.*, 2005), thus data based on counts can be flawed. In addition, the mathematical model of Faddy *et al*. on which Johnson *et al*. based their assumption that 77 new follicles would be required daily could have led to an overestimate of the shortfall in follicle numbers, as (a) there may be differences between the different strains of mice used by the two groups, and (b) the rate of decline in follicle numbers changes with age; therefore Faddy *et al.*'s estimate of primordial follicle depletion may not be applicable to all stages of reproductive life (Faddy *et al.*, 1987, Johnson *et al.*, 2004, Gosden, 2004, Greenfeld and Flaws, 2004). It should be noted, however, that Faddy *et al*. performed counts over a 100 day period, and therefore their analysis should take account of much of the age-related changes in the rate of follicle decline (Faddy *et al.*, 1987).

In addition, indirect evidence of *neo*-oogenesis using follicular quantification, mathematical modelling and lineage tracing techniques (Johnson *et al.*, 2004, Kerr *et al.*, 2006, Reizel *et al.*, 2012) has been contradicted by other groups. By comparing follicle counts in mice from 6 days post-natal to 12 months of age, it has been reported that follicle development dynamics indicate that the ovarian reserve is not supplemented by post-natal *neo*-oogenesis (Bristol-Gould *et al.*, 2006). Indeed, in this study, the fixed ovarian reserve mathematical model was able to replicate the rise and fall in preantral follicles that is observed over time, whereas the stem cell model did not correspond to the same pattern in the mouse (Bristol-Gould *et al.*, 2006). This was despite attempting to integrate Johnson *et al*.'s assertion that 77 new follicles per day are required to be formed to sustain the follicular pool (Johnson *et al.*, 2004). The authors reported that much lower follicular production rates (i.e. less than 10 new follicles daily) *are* compatible with their experimental findings, but that this was within the error margin of their counting methodology. This research has been supported in humans by Wallace and Kelsey's modelling work, which demonstrated

that the follicular kinetics of a theoretical germline stem cell model does not fit with that seen *in vivo* (Wallace and Kelsey, 2010). It has been suggested that for Johnson *et al*.'s (Johnson *et al.*, 2004) and Kerr *et al*.'s (Kerr *et al.*, 2006) assertions regarding follicular counts to be regarded as reliable, much bigger sample sizes would be required (Faddy and Gosden, 2007).

Related to the subject of follicular dynamics, research using a knock-out mouse model for Foxo3 has implied that post-natal *neo*-oogenesis does not occur (John *et al.*, 2007). Due to Foxo3's role in inhibiting primordial follicle activation, this strain of mice exhibits universal follicular activation shortly after birth, leading to POI (Castrillon *et al.*, 2003). Interestingly, all other aspects of germ cell development and folliculogenesis are unaffected by the knock-out (John *et al.*, 2007), indicating that, should *neo*-oogenesis exist, follicles should continue being formed and ovulated throughout life and POI would not occur.

Although lineage tracing has provided support for post-natal *neo*-oogenesis (Reizel *et al.*, 2012), this technique has provided conflicting data with a separate study presenting indirect evidence in support of the fixed ovarian reserve dogma. Using a tamoxifen-inducible Cre system in mice, germ cells were labelled with YFP and their behaviour over time was observed (Lei and Spradling, 2013). The investigators found that the turnover of primordial follicles was very low, with follicles being extremely stable, exhibiting a half-life of 10 months. Furthermore, the authors never detected any evidence of YFP-labelled germ cell clusters, which they believed would be indicative of OSC proliferation [although other researchers have reported the existence of such clusters in OSE scrapings in adult mice and sheep (Bhartiya *et al.*, 2013)]. Zhang *et al*. also used an inducible Cre system, in which Gdf-9-positive cells (i.e. oocytes) were selectively ablated in adult mice (Zhang *et al.*, 2014). After complete ablation of the ovarian reserve, no new oocytes were detected up to 1 year following induction of the Cre system.

With respect to the cells that Johnson *et al*. postulated were OSCs (Johnson *et al.*, 2004), sceptics suggested that the presence of BrdU in the OSE-located, Ddx4-positive cells may be due to mitochondrial DNA (mtDNA) synthesis rather than evidence of cellular proliferation (Notarianni, 2011). Certainly, Zuckerman believed that:

> "mitotic activity in the adult germinal epithelium is not necessarily indicative of oogenesis" (Zuckerman, 1951).

Alternative explanations also have been proposed for the observed behaviour of OSCs in culture. It has been suggested that the reason OSCs appear to take much longer to establish in culture than SSCs may be as a result of *in vitro* transformation (Oatley and Hunt, 2012). However, given the relative paucity of research into OSCs in comparison to SSCs, it is difficult to make such comparisons between the two cell types at this time. Another critique is that spontaneously *in vitro* generated OLCs may be cells undergoing oncosis rather than oogenesis (Notarianni, 2011).

The finding of GFP-positive oocytes surrounded by wild-type somatic cells (Johnson *et al.*, 2004) when GFP-positive grafts are transplanted onto wild-type ovaries has been the subject of scepticism, with questions raised over why wild-type oocytes surrounded by GFP-positive somatic cells were not also detected (Greenfeld and Flaws, 2004). It has been suggested that these follicles are a result of ovarian plasticity rather than *neo*-oogenesis (Gosden, 2004, Byskov *et al.*, 2005), as disaggregated ovaries can reassemble into follicles that can ovulate (Gosden, 1990). This indicates that the damage caused during grafting may cause "reshuffling" of cells between the two strains of tissue, with GFP-positive oocytes recombining with wild-type somatic cells (Gosden, 2004). Studies have shown that injection of GFP-expressing fetal ovarian cells into the ovaries of post-natal wild-type mice do result in the formation of GFP-expressing follicles, but both the oocyte and the somatic cells fluoresce (Zhang *et al.*, 2012). In other words, the adult ovary supported the development of, but did not contribute to, *neo*-folliculogenesis (Zhang *et al.*, 2012). However, it should be noted that the ovarian cells that were injected were not purified in any way and therefore it is unclear whether the results would have been different if a solely OSC population had been used. Concerns have also been raised that the GFP expression seen in oocytes may be a result of transfer from other cells, perhaps by cell fusion, rather than evidence of the cells being *de novo* oocytes (Byskov *et al.*, 2005, Telfer *et al.*, 2005, Vogel, 2012).

Results which suggested that putative OSCs, or their precursors, are blood-borne due to germline markers such as *Pou5f1*, *Ddx4* and *Ifitm3* being present in the blood and bone marrow (Johnson *et al.*, 2005a) have been especially criticised, with the argument

made that these markers should not be considered to be germ cell-specific (Telfer *et al.*, 2005). It is possible that the findings merely indicate the permissiveness of blood and bone marrow cells rather than *bona fide* OSCs. The assertion that patients who receive bone marrow transplants do not acquire a restoration of ovarian activity (Telfer *et al.*, 2005) was rapidly counteracted by the Tilly group who cited several instances where women had conceived following BMT despite previously suffering POI (Johnson *et al.*, 2005b). Furthermore, a post-menopausal patient who underwent heterotopic reimplantation of cryopreserved ovarian tissue to her anterior abdominal wall has spontaneously conceived on four occasions from her native ovaries (Oktay *et al.*, 2011). The mechanism underlying this remains unclear: she may still have had some functional oocytes in her native ovaries despite her clinical and biochemical diagnosis of POI, as seen in a separate case where spontaneous conception occurred in a woman who had apparently undergone POI secondary to chemo- and radiotherapy (Bath *et al.*, 2004). However, another hypothesis is that *neo*-oogenesis occurred in the previously menopausal ovaries (Oktay, 2006), perhaps triggered by the heterotopic transplant.

Another line of argument is that, if OSCs exist, how have they gone unnoticed for so long (Telfer *et al.*, 2005)? They have, however, been demonstrated to be a remarkably rare population, which apparently declines with age (Pacchiarotti *et al.*, 2010, White *et al.*, 2012), therefore perhaps it has only become possible to detect them following the advent of new technologies. A related line of questioning is why does reproductive senescence occur if OSCs are present (Greenfeld and Flaws, 2004, Oatley and Hunt, 2012)? The counterargument is twofold: (1) OSCs are likely to undergo the same detrimental ageing process as other cells and thus lose their ability to differentiate into oocytes, and (2) an aged stromal environment may be inhibitory to oogenesis. Many other stem cells, including haematopoietic, neural and skeletal muscle stem cells, lose function during the mammalian lifespan (Signer and Morrison, 2013) and therefore OSCs may not be immune from the ageing process. Indeed, in *Drosophila*, where lifelong *neo*-oogenesis is an accepted phenomenon, fGSC proliferation and thus oogenesis declines with age (Zhao *et al.*, 2008). In addition to reduced oocyte production, there is also increased oocyte apoptosis, the incidence of which increases with age (Zhao *et al.*, 2008). It has been reported that this observation coincides with

a decrease in important signalling pathways (including BMP signalling) in the germ cell niche which may have a detrimental effect on fGSC mitosis (Pan *et al.*, 2007, Zhao *et al.*, 2008). Therefore, both the intrinsic nature of the cell itself and its extrinsic environment are important in maintaining stem cell function. As previously discussed, mouse OSC prevalence decreases with age (Pacchiarotti *et al.*, 2010) and the function of aged OSCs only resumes when transplanted back into a young stromal environment (Niikura *et al.*, 2009), indicating that reproductive senescence and post-natal oogenesis are not necessarily mutually-exclusive.

1.2.5.2 The Use of DDX4 for Isolation of Putative OSCs

Perhaps the most controversial area of debate concerns the use of Ddx4/DDX4 to select putative OSCs. DDX4 is considered to be a specific marker of PGCs and developing oocytes within the ovary and is generally believed to be intracytoplasmic in location (Castrillon *et al.*, 2000). As sorting methods for intact cells require the use of cell surface markers to which antibodies can bind, it does not therefore seem possible that DDX4 could be utilised in this manner. However, it has been postulated that the protein has an external epitope in OSCs, which is internalised in more mature germ cells (White *et al.*, 2012; Fig. 1.7), as bioinformatics has suggested that the protein does in fact have transmembrane-spanning domains, thus justifying its use (Abban and Johnson, 2009, Zou *et al.*, 2009). A study has demonstrated that some cells do express DDX4 on the cell surface: a porcine cell line transfected with a DDX4-containing plasmid expressed DDX4 on its cell surface as confirmed by flow cytometry, showing that it is possible for DDX4 to be expressed externally (Kakiuchi *et al.*, 2014). Furthermore, flow cytometry of disaggregated pre-pubertal porcine testes detected a population of small cells with the DDX4 antibody used by White *et al*. (White *et al.*, 2012) which expressed *PRDM1* and *IFITM3*, although they did not appear to form germ cells and the authors concluded that they were not GSCs (Kakiuchi *et al.*, 2014). Further information on the nature of the external epitope has come from White *et al*.'s study which demonstrated that only an antibody against the C-terminus of the protein is capable of selecting a Ddx4-positive cell population, whereas Ddx4-positive cells can only be detected by a N-terminus antibody if the cells are permeabilised (White *et al.*, 2012).

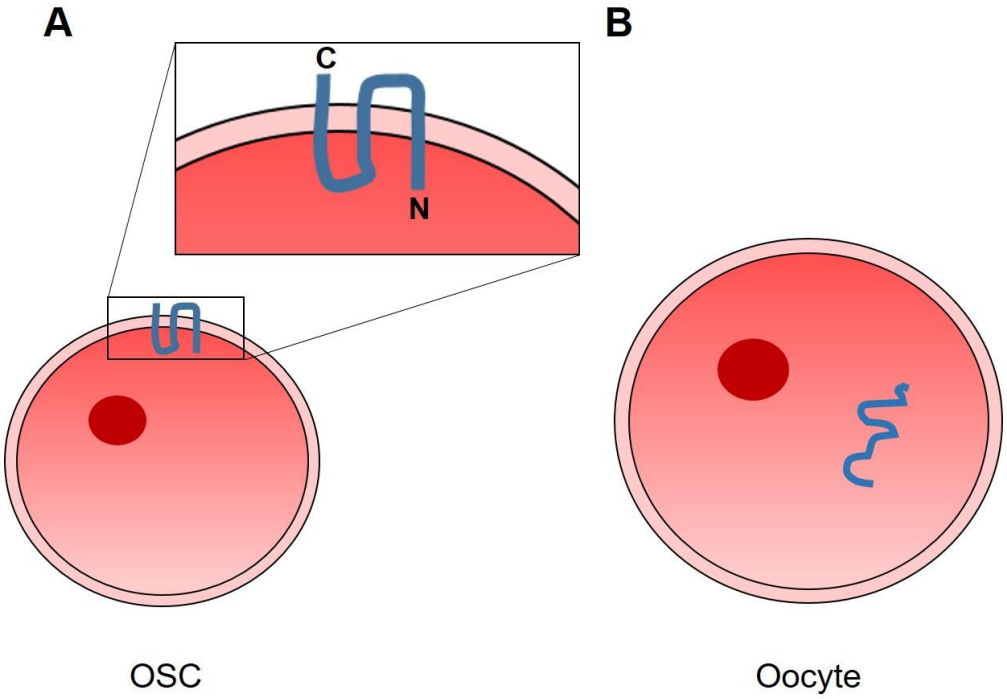


Figure 1.7. DDX4 cellular localisation. (A) It is postulated that, in OSCs, DDX4 has transmembrane domains such that the C-terminus is extracellular (White *et al*., 2012), before being internalised in **(B)** oocytes where it is found to be intracytoplasmic (Castrillon, 2000).

Yet, conflicting data has been reported demonstrating that Ddx4/DDX4 is not expressed by putative mouse and human OSCs (Zhang *et al.*, 2012, Hernandez *et al.*, 2015). Zhang *et al*. used a fluorescent germline reporter mouse (*Rosa26*$^{rbw/+}$*;Ddx4-Cre*) in which Ddx4-expressing cells change from fluorescing green to fluorescing either red (RFP), orange (OFP) or cyan (CFP) (Zhang *et al.*, 2012). This allows the Ddx4-positive cells to be traced. No proliferation of isolated, *in vitro* cultured female RFP-expressing cells was found over 72 hours, in contrast to male cells which mitosed 1-3 times; indeed, these female cells did not establish in culture at all. However, as the cells were selected only on their expression of RFP and no further characterisation of these cells was apparently performed, it is possible the cells the authors were examining were oocytes, which clearly would not be expected to proliferate (Woods

et al., 2013). In reply, Tilly's group subsequently used a similar transgenic mouse strain (*Rosa26*$^{tdTm/tdTm}$*;Ddx4-Cre*) whereby Ddx4 promoted the expression of *tomato red* (*tdTM*) to repeat the proliferation analysis experiment and demonstrated that oocytes were indeed included in the likely cell population that Zhang *et al*. examined (Zhang *et al.*, 2012, Park and Tilly, 2015). They also demonstrated that a subpopulation of tdTM-positive cells could be isolated during FACS using a DDX4-antibody and this population behaved like OSCs; e.g. they established in culture and expressed germline markers (*Prdm1*, *Dppa3*, *Ifitm3*, *Ddx4*) (Park and Tilly, 2015).

However, despite using the same protocol as White *et al*. (White *et al.*, 2012, Woods and Tilly, 2013), and identifying apparently Ddx4/DDX4-positive populations by flow cytometry, three groups have been unable to demonstrate Ddx4/DDX4 expression at either the mRNA or protein level, nor by mass spectrometry in freshly isolated or cultured cells (Hernandez *et al.*, 2015, Zhang *et al.*, 2015, Zarate-Garcia *et al.*, 2016), although Zhang *et al*. only analysed a small number of cells (n = 38) by mRNA sequencing (Zhang *et al.*, 2015). Furthermore, the mouse cells that Zarate-Garcia *et al*. isolated died after 2-3 months (Zarate-Garcia *et al.*, 2016), calling into question whether they isolated the correct population. Hernandez *et al*. thus investigated the specificity of the polyclonal antibody utilised in the protocol by transducing human OSCs with a lentivirus containing DDX4 with a "tag" at the C-terminus (Hernandez *et al.*, 2015). When FACS was performed using the DDX4-antibody and an antibody against the tag, an apparently DDX4-positive population was identified, but no tag-positive population was seen. However, if the cells were permeabilised, both DDX4- and tag-positive cells were seen. Zhang *et al*. reported that cultured cells did not express DDX4 on immunofluorescence, but that the DDX4-antibody used by White *et al*. (White *et al.*, 2012) did detect a population of "DDX4-positive" cells (Zhang *et al.*, 2015). Moreover, Zarate-Garcia *et al*. have disputed the bioinformatics finding that DDX4 has a transmembrane location (Abban and Johnson, 2009, Zou *et al.*, 2009), by providing *in silico* evidence that several other computer-based programmes do not predict an external epitope for the protein (Zarate-Garcia *et al.*, 2016). These findings have led to the conclusion that the DDX4-antibody is detecting another protein and is not specific: as such, sorting based on DDX4 has been deemed inadequate by some in the field (Albertini and Gleicher, 2015, Hernandez *et al.*, 2015, Zhang *et al.*, 2015,

Zarate-Garcia *et al.*, 2016). However, Tilly's group defended the use of DDX4, suggesting that technical constraints may be the reason for the evident lack of DDX4 expression (Woods and Tilly, 2015). Furthermore, a monoclonal DDX4 antibody has also been used to isolate OSCs (Fakih *et al.*, 2015) and the sequence targeted by the C-terminus antibody is only homologous with a sequence in ATP-binding cassette subfamily C member 12 (ABCC12) that is located intracellularly (Woods and Tilly, 2013).

However, although the use of DDX4 is contentious, OSCs have been isolated using other putative markers (e.g. Ifitm3 (Zou *et al.*, 2011, Xiong *et al.*, 2015, Lu *et al.*, 2016)); therefore the DDX4 argument alone is not sufficient to prove that OSCs do not exist.

1.2.5.3 Reproducibility of the Data

Reproducibility of data is a fundamental aspect of science in order that results are deemed reliable. Studies on all types of stem cells are proving particularly challenging to replicate, with technical difficulties, or methodological differences, often at the root of the problem (Check, 2007). Research on OSCs has proved no exception: the problems with repeating the DDX4-based isolation have already been discussed (section 1.2.5.2) and Tilly reported that it took many months for his group to recapitulate the isolation of mouse OSCs reported by Zou *et al*. (Zou *et al.*, 2009, Vogel, 2012). Some other reproducibility issues are now detailed below.

Firstly, the detection of proliferation within the mouse OSE (Johnson *et al.*, 2004) was contradicted by mouse, monkey and human data which found no evidence of mitotic markers (Ki-67 and PCNA) within the adult ovary (Liu *et al.*, 2007, Yuan *et al.*, 2013). Moreover, the discovery that genes of early meiosis (e.g. Sycp3, Dmc1) were detectable in post-natal mouse ovaries (Johnson *et al.*, 2004, Zhang *et al.*, 2008) was also challenged by these studies, with no mRNA or protein evidence of such genes being identified (Liu *et al.*, 2007, Yuan *et al.*, 2013).

Two murine experiments have been unable to reproduce Johnson *et al*.'s (Johnson *et al.*, 2004) findings that OSCs are blood-borne (Eggan *et al.*, 2006, Begum *et al.*, 2008). In the first experiment, two mice (one wild-type and one transgenic mouse which

ubiquitously expressed GFP) were surgically linked (parabiosis) to share a circulatory system (Eggan *et al.*, 2006). It was hypothesised that if OSCs were indeed present in the bloodstream, then GFP-positive oocytes would be found in the wild-type mouse's ovaries and vice versa. Upon superovulation, MII oocytes were collected and examined, revealing no oocyte chimaerism: i.e. wild-type mice only ovulated non-GFP-expressing oocytes, whilst GFP mice ovulated only GFP-expressing oocytes. This remained true if the wild-type mouse underwent gonadotoxic treatment, ruling out the theory that the ovaries may need to experience an injury to allow circulating OSCs to embed and undergo folliculogenesis. Finally, the investigators performed a GFP-positive BMT on wild-type, sterilised mice and reported that no ovulated oocytes were GFP-positive (Eggan *et al.*, 2006). This appeared extremely convincing, although only ovulated, mature oocytes were examined, whereas Tilly's group examined solely immature follicles within the ovary (Johnson *et al.*, 2005a, Lee *et al.*, 2007). The second experiment involved grafting wild-type ovaries, some of which were irradiated, into GFP-expressing mice and subsequently analysing the graft for GFP-positive oocytes (Begum *et al.*, 2008). None were detected, thus once again refuting the theory that OSCs are found within the circulation.

Proof that the cells in question can undergo oogenesis and create healthy offspring is clearly essential if it is to be believed that they are OSCs. With the exception of two groups whose work has demonstrated the creation of live mouse and rat offspring from OSCs (Zou *et al.*, 2009, Zhou *et al.*, 2014, Lu *et al.*, 2016), evidence that the putative OSC populations described by other groups can develop into healthy, competent, fertilisable oocytes is lacking. Using White *et al*.'s isolation and human xenograft protocol (White *et al.*, 2012), Zhang *et al*. failed to replicate the findings of GFP-positive oocytes in human ovarian cortex grafted into immunodeficient mice up to 4 weeks following grafting (Zhang *et al.*, 2015). Furthermore, the authors demonstrated that sorted cells from red-fluorescing (Tomato) mice did not undergo *neo*-oogenesis when injected into the ovaries of chemoablated mice (Zhang *et al.*, 2015).

Clearly, there are very distinct viewpoints on the subject of *neo*-oogenesis and OSCs in female mammals; however, overall, the data available suggests that a population of

cells exist that, under the right conditions, may be able to undergo oogenesis. It remains uncertain whether OSCs have a physiological role in the "normal" ovary, although very recent research suggests they might, at least in the mouse (Guo *et al.*, 2016). Thus, it is undoubtedly an interesting and exciting area of research that is worthy of study.

1.2.6 The Capacity of other Stem Cells to form Oocytes

A separate population of ovarian stem cells has also been reported to undergo oogenesis in specific environments. Named by some as very small embryonic-like (VSEL) stem cells, these cells are collected from scrapings of the OSE and are smaller than the putative OSCs discussed previously, being less than 4 µm in diameter (Virant-Klun *et al.*, 2008). They were first described in post-menopausal women and women suffering POI: collected cells expressed pluripotency markers (including *POU5F1, NANOG* and *SSEA-4*) and generated large OLCs *in vitro*, although their developmental competency was not tested (Bukovsky *et al.*, 2005, Virant-Klun *et al.*, 2008, Parte *et al.*, 2011). Counterpart cells have been identified in the adult rabbit, sheep and marmoset monkey (Parte *et al.*, 2011). Virunt-Klun *et al*. went on to purify these cells using immunomagnetic and FACS sorting for the presence of SSEA-4 and demonstrated that they express similar markers to OSCs, including *LIN28*, *PRDM1* and *DPPA3* (Virant-Klun *et al.*, 2013). Parte *et al*. actually detected a second putative stem cell population, larger in size and which could be considered analogous to OSCs (Parte *et al.*, 2011). It has therefore been proposed that VSELs are precursors to OSCs (Parte *et al.*, 2011), although the reported existence of VSELs has also attracted controversy (Notarianni, 2011).

Other non-ovarian stem cells have also been reported to have the ability to produce oocytes. Both ESCs and iPSCs can be induced to become primordial germ cell-like cells (PGCLCs) in mice in a two-step manner, by first exposing the cells to activin A and basic fibroblast growth factor (bFGF) and then culturing the cells in the presence of other growth factors including BMP4, BMP8a and epidermal growth factor (EGF) (Hayashi *et al.*, 2012). Following aggregation of these PGCLCs with developmentally-appropriate fetal gonadal somatic cells and transplantation under the ovarian bursa of immunodeficient mice, oocytes at the germinal vesicle stage could be

collected, matured *in vitro* and fertilised to produce live, normally imprinted pups (Hayashi *et al.*, 2012). This research highlights the importance of the stem cell niche in permitting normal development of the oocyte. Human ESC research has resulted in the production of follicle-like structures, but the oocytes did not possess a zona pellucida (Aflatoonian *et al.*, 2009).

However, human endometrial mesenchymal cells (EnSCs) found in menstrual blood have been found to rescue the fertility of chemoablated rodents (Lai *et al.*, 2015). GFP-expressing EnSCs injected into the tail vein of sterilised mice were detected within the ovaries 2 months after transplantation. Although they appeared to differentiate into granulosa cells rather than OSCs, with expression of FSH receptor, the authors postulated that they support OSC renewal, as transplanted chemo-ablated mice had more DDX4-BrdU dual-stained cells (deemed OSCs) than non-transplanted chemo-ablated mice (Lai *et al.*, 2015).

Moreover, human amniotic fluid stem cells (hAFSCs) have been induced by the presence of PFF to form OLCs, which coincided with upregulation of *BMP15* expression and that of other oocyte markers, including *ZP2* and *3* and *DDX4* (Cheng *et al.*, 2012). A human hepatic cell line (Ma *et al.*, 2013) and fetal porcine skin cells (Linher *et al.*, 2009) have also been reported to undergo differentiation into a distinct cell type *in vitro* which expresses pluripotency (e.g. *POU5F1*) and germline (e.g. *DPPA3*, *PRDM1*, *DDX4*) markers and, during prolonged culture, these cells can form OLCs (Linher *et al.*, 2009) or follicle-like structures containing OLCs (Ma *et al.*, 2013). None of these types of cells have been fertilised and therefore their reproductive potential is as yet unknown.

An advantage that OSCs may have over these other types of cells is that they are postulated to be unipotent and thus already further down the differentiation pathway than, for example, ESCs. This means that induction to form oocytes may not take as many steps, or factors, and thus may be faster and easier. Moreover, the use of OSCs bypasses the ethical issues surrounding the destruction of embryos for the collection of ESCs.

1.3 Hypotheses

Given the importance of reproducibility in science, the controversy surrounding the concept of OSCs and the great potential such a cell population could have scientifically and clinically, it was important to investigate whether the isolation of putative OSCs could be recapitulated. At the time of commencing the research within this thesis, putative OSCs had only been isolated in mice (Zou *et al.*, 2009, Pacchiarotti *et al.*, 2010, Zou *et al.*, 2011, Hu *et al.*, 2012, White *et al.*, 2012) and, by one group, in humans (White *et al.*, 2012) and therefore this research concentrated on repeating the isolation of human OSCs and attempting the isolation of putative OSCs in a large animal model, namely the cow. Furthermore, if such a population of cells were to exist, it would be imperative to test their functional capabilities.

Our hypotheses therefore were that a population of mitotically active cells with germline potential (i.e. OSCs) could be isolated from human and bovine ovarian cortex and cultured long-term *in vitro*. Secondly, that these cells could be shown to undergo *neo*-oogenesis and demonstrate potential as a germ cell model.

1.4 Aims

In order to test the hypotheses, the following specific aims were devised:

1. To corroborate independently the existence of OSCs by isolating and purifying cells from bovine and human ovarian cortical tissue using a previously validated approach (White *et al.*, 2012).
2. To characterise these cells and determine whether they demonstrate stem cell and germline-specific gene expression at the mRNA and protein level.
3. To use *in vitro* culture systems to attempt to support *neo*-folliculogenesis and produce mature oocytes, characterised using oocyte- and meiosis-specific markers to examine their viability.
4. To investigate the potential of the cells as a model for germ cell development by exposing them to key regulators of the germ cell differentiation pathway.
5. To develop a novel system to deliver cells into adult ovarian cortex.

Chapter 2

General Materials and Methods

Standard laboratory protocols from Prof. Richard Anderson's and Prof. Evelyn Telfer's laboratories were used for all methods unless otherwise specified. Non-sterile glassware and plastics were autoclaved prior to use by support staff at the Centre for Reproductive Health and Centre for Integrative Physiology, University of Edinburgh. Manufacturers' locations can be found in Appendix 1.

2.1 Tissue Collection

2.1.1 Bovine Ovarian Tissue

Post-mortem adult bovine ovaries from freshly killed heifers were collected by Mr. John Binnie (technician in Prof. Telfer's laboratory) from a local abattoir (Bridge of Allan, UK) and transported to the laboratory in HEPES-buffered M199 medium (Life Technologies) supplemented with amphotericin B (2.5 µg/ml; Life Technologies), pyruvic acid (25 µg/ml), penicillin G (75 µg/ml) and streptomycin sulphate (50 µg/ml; all chemicals from Sigma-Aldrich). Thin slices of ovarian cortex were aseptically removed using a scalpel under a laminar flow hood, placed in a glass Petri dish and immersed in dissecting medium consisting of Leibovitz L-15 supplemented with sodium pyruvate (2mM), glutamine (2mM; all Life Technologies), bovine serum albumin (BSA; 3mg/ml), penicillin G (75 µg/ml) and streptomycin sulphate (50 µg/ml; all from Sigma-Aldrich). These slices were examined under light microscopy and any medullary tissue was removed before the tissue was cut into approximately 1cm x 1cm x 1mm pieces. Approximately two pieces from each abattoir collection were fixed immediately for 24 hours in 4% neutral buffered formalin (NBF). The remaining ovarian cortical pieces were immediately vitrified and cryopreserved as described in section 2.2.

Fetal bovine ovaries were collected from the same abattoir. The reproductive organs from two fetuses were retrieved and transported to the laboratory in the transport medium detailed above. Ovaries were dissected from the fallopian tubes and either snap frozen or subjected to enzymatic dissociation. Both ovaries from a 166 day old fetus (estimated by measurement of crown-rump length (CRL) = 44cm) were snap frozen for subsequent use as positive controls in reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blotting. The ovaries were placed in 1.5ml

microfuge tubes (Eppendorf) and immediately placed on dry ice for 10 mins before being transferred to a -80ºC freezer. Both ovaries from a 164 day old fetus (CRL = 43cm) were enzymatically dissociated as described in Chapter 5.

2.1.2 Human Ovarian Tissue

Post-pubertal ovarian biopsies were performed on seven adult women (22-40 years old) and one post-pubertal teenager (13 years old) (Table 2.1). Two women and the teenager were undergoing laparoscopic ovarian tissue removal for cryopreservation for the purposes of fertility preservation. Both adults had previously received chemotherapy, whilst the teenager had her tissue removed prior to cancer therapy. The remaining 5 adults had ovarian tissue removed during elective Caesarean section. All women were recruited by Research Nurses, Joan Creiger and Anne Saunderson, gave informed written consent and the study received Lothian Research Ethical Committee approval (reference number: REC10/S1101/24).

The ovarian pieces were placed in dissecting medium (described above) and, using a light microscope, medullary tissue was aseptically removed with a scalpel under a laminar flow hood. The cortex was then cut into approximately 0.5cm x 0.5cm x 1mm pieces and vitrified as described in section 2.2. One piece of cortex from each patient was fixed for 24 hours in 4% NBF and the remaining pieces were vitrified as described in section 2.2.

Table 2.1. Characteristics of patients who consented to use of their ovarian tissue for the purposes of research. ABVD = adriamycin, bleomycin, vinblastine and dacarbazine; FEC = fluorouracil, epirubicin, cyclophosphamide).

Patient	Age (years)	Pathology	Procedure During which Sample Taken
1	13	Ewing's sarcoma (pre-treatment)	Laparoscopic ovarian cortical stripping for cryopreservation
2	22	Hodgkin's Lymphoma (relapse; previous ABVD chemotherapy)	Laparoscopic ovarian cortical biopsies for cryopreservation
3	32	N/A	Elective Caesarean Section
4	33	Previous breast cancer (BRCA2 mutation; previous taxol and FEC chemotherapy)	Prophylactic bilateral oophorectomies
5	33	N/A	Elective Caesarean Section
6	38	N/A	Elective Caesarean Section
7	39	N/A	Elective Caesarean Section
8	40	N/A	Elective Caesarean Section

Fetal human ovarian tissue was collected from morphologically normal second trimester fetuses (16-19 weeks gestation) after medical termination of pregnancy. Recruitment was performed by Research Nurses, Joan Creiger and Anne Saunderson, written informed consent was obtained from the mothers and the study received Lothian Research Ethical Committee approval (LREC08/S1101/1). The gestation of the fetuses was calculated from ultrasound findings prior to the termination and confirmed by foot length measurement in the lab. Sex of the fetus was determined by visualisation of the external genitalia and dissection of the ovaries was performed by Dr. Rosey Bayne (from Prof. Anderson's group) under sterile conditions. The ovaries were dissected from attached mesonephros tissue using sterile 25G needles and placed in Dulbecco's Phosphate-Buffered Saline (DPBS; Life Technologies). They were then

either snap frozen for subsequent use in RT-PCR and Western blotting or enzymatically dissociated as per Chapter 5.

2.2 Tissue Vitrification and Thawing

2.2.1 Vitrification

After dissection and cutting, ovarian cortical pieces were immediately vitrified and cryopreserved as previously described (Kagawa *et al.*, 2009). Cortical pieces were equilibrated at room temperature for 25 minutes in a holding medium comprising Leibovitz L-15, sodium pyruvate (2mM), glutamine, penicillin G (75 µg/ml), streptomycin sulphate (50 µg/ml), 20% (weight/volume; w/v) fetal bovine serum (FBS; Life Technologies), and supplemented with 7.5% (volume/volume; v/v) ethylene glycol (EG) and 7.5% (v/v) dimethyl sulphoxide (DMSO, both Sigma-Aldrich). The pieces were then placed in a second equilibration medium consisting of the above holding medium supplemented with 20% (v/v) EG, 20% (v/v) DMSO and 0.5 mol/L sucrose; Sigma-Aldrich) for 15 minutes at room temperature. Fine forceps were subsequently used to thread the pieces onto sterilised stainless steel acupuncture needles (Acumedic®) and these needles were submerged briefly in liquid nitrogen. Once the tissue was frozen, the needles were placed in cryovials and stored in the vapour phase of liquid nitrogen.

2.2.2 Thawing

Cryovials containing cryopreserved ovarian cortical pieces were removed from the liquid nitrogen container and transported to a laminar flow hood on dry ice. The protocol described by Kagawa *et al.* (Kagawa *et al.*, 2009) was again used. The acupuncture needles holding the tissue were removed and both needles and tissue were placed in a thaw medium for 1 min at 37ºC. The thaw medium comprised the holding medium described in section 2.2.1, supplemented with 1.0 mol/L sucrose. The needles were then transferred to a second thaw medium consisting of the holding medium supplemented with 0.5 mol/L sucrose and incubated for 5 mins at room temperature. The pieces of tissue were then removed from the needles using fine forceps and washed

twice in holding medium for 10 mins per wash. The tissue was then ready for use in experiments.

2.3 Isolation of Putative OSCs

Ovarian cortex was dissociated, prepared and sorted for DDX4-positive cells as per the protocol previously published by Tilly's group (White *et al.*, 2012, Woods and Tilly, 2013) with some modifications.

2.3.1 Ovarian Cortex Dissociation

Vitrified bovine and human ovarian cortex was thawed as per section 2.2 before being mechanically and enzymatically disaggregated. Approximately 15 pieces of bovine tissue and 2 - 4 pieces of human tissue were used for each experiment. Cortical pieces were placed in 1ml of 600u/ml (bovine) or 400u/ml (human) collagenase type IV (Worthington Biochemical Company) pre-warmed to 37ºC and supplemented with DNase I (stock concentration of 1mg/ml; Roche) at a 1:100 DNase I:collagenase ratio in Hank's Balanced Salt Solution (HBSS) minus calcium (Ca^{2+}) and magnesium (Mg^{2+}); Life Technologies). Varying concentrations of collagenase type IV (400u/ml to 800u/ml) and DNase I:collagenase ratios (1:1000 to 1:100) were trialled to determine the optimal enzymatic conditions. The pieces were cut into approximately 1mm x 1mm x 1mm fragments with scalpels in a glass Petri dish, transferred into a gentleMACS™ C tube (Miltenyi Biotec) and a further 4ml of collagenase:DNase mixture added. The tube was then placed in a gentleMACS™ Dissociator (Miltenyi Biotec) and mechanically dissociated using the pre-set "h_tumor_01.01 "h_tumor_02.01" and "h_tumor_03.01" programmes successively. In between programmes, tissue fragments that were trapped in the gentleMACS™ C tube rotor were placed back in the enzyme solution using sterile forceps within a laminar flow hood and the tube was rotated in a MACSmix™ Tube Rotator (Miltenyi Biotec) on its highest setting for 20 min incubations at 37ºC / 5% CO_2. Once the "h_tumor_03.01" programme was completed, the sample was removed from the tube by a sterile 10ml glass pipette (Fisher Scientific) and filtered into a 15ml centrifuge tube (Fisher Scientific) through a 100µm filter (Partec CellTrics®, Partec). The filter was soaked

in Leibovitz L-15 supplemented with 10% (v/v) FBS prior to use to help prevent cell adherence to the filter. The gentleMACS^{TM} C tube was washed with 10ml HBSS minus calcium (Ca^{2+}) and magnesium (Mg^{2+}) warmed to 37ºC and this solution was also passed through the filter. The sample was centrifuged at 300 rcf for 5 mins at room temperature with the brake off, the supernatant was carefully removed and the cell pellet was resuspended in 10ml of warmed HBSS as a wash step. The sample underwent repeat centrifugation at the above specifications and the cell pellet was then blocked in 500µl blocking solution (2% (v/v) normal goat serum (Life Technologies) and 2% (w/v) BSA in HBSS minus Ca^{2+} and Mg^{2+}, sterile-filtered through a 0.2µm filter (Corning)) for 20 mins at 4ºC. Fifty to one hundred microlitres of the sample was placed into two other 15ml tubes to be used as a negative control (both primary and secondary antibodies omitted) and a secondary antibody only control (with only the primary antibody omitted). The cell suspensions were kept at 4ºC from this point onwards and used in the FACS protocol detailed below in section 2.3.2.

2.3.1.1 Alternative ovarian cortex dissocation protocol

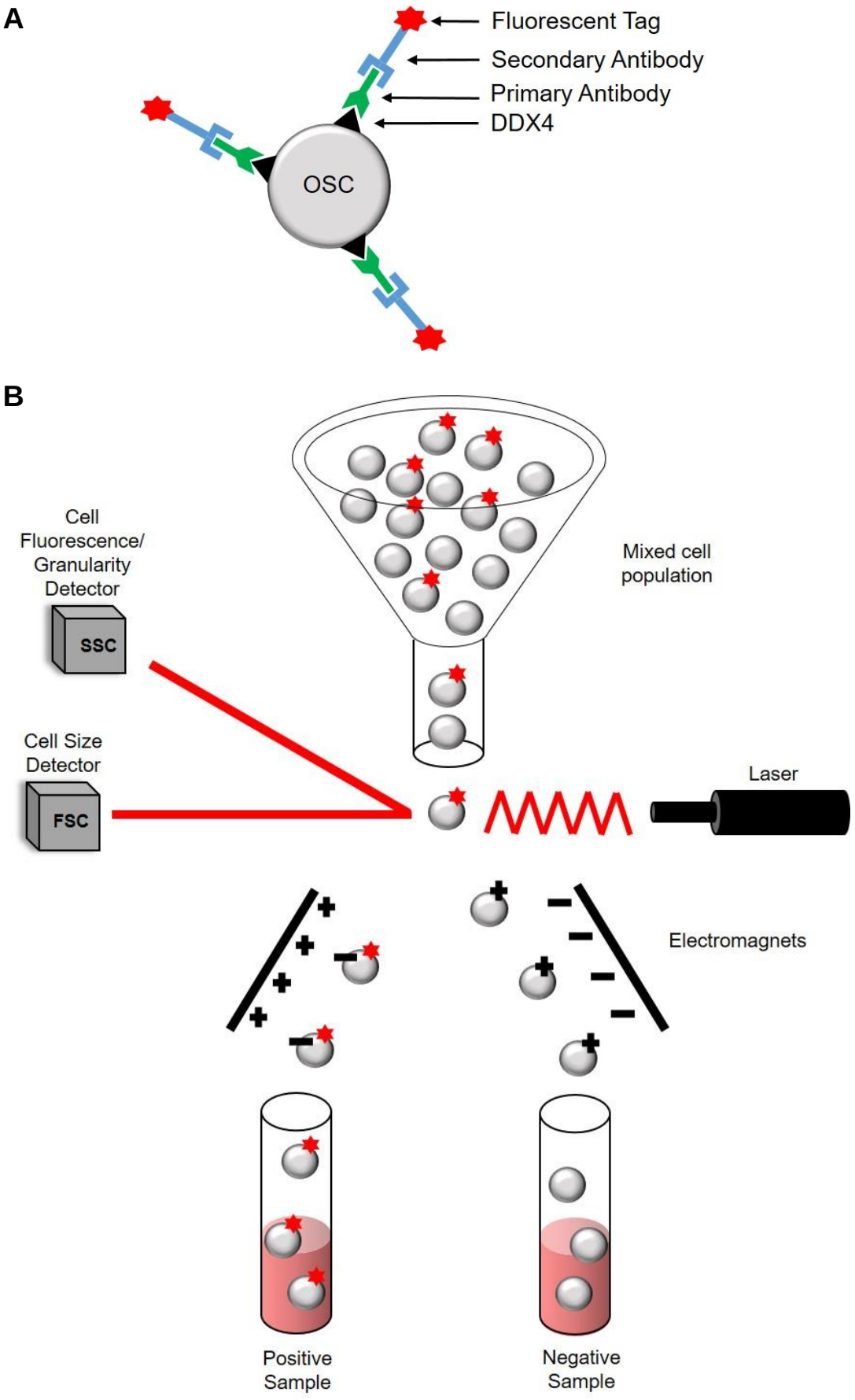
Due to the low cell yields and high cell death rates achieved using this dissociation protocol, post-doctoral colleagues in Prof. Telfer's group (Dr. Marie McLaughlin and Dr. Yvonne Clarkson) investigated further adaptations of the disaggregation aspects of the protocol aiming to improve the number of live cells used in flow cytometry. Their optimised protocol was subsequently utilised by myself to collect higher cell yields for the purposes of characterisation of freshly isolated cells. This protocol entailed thawing the tissue as per section 2.2 and cutting the tissue with scalpels as above, but in the absence of enzymes. Tissue was then placed into gentleMACS^{TM} C tubes and an enzyme combination of 400 u/ml collagenase I/II (Roche) and 10.9 u/ml DNase I in warmed HBSS *plus* Ca^{2+} and Mg^{2+} (to potentiate the enzyme effect; Life Technologies). The gentleMACS^{TM} Dissociator steps were followed as above, but the incubation steps inbetween were omitted, thus reducing the exposure time of the tissue to the enzymes with the aim of reducing resultant cell death. The cell pellet was then blocked and divided into separate 15ml tubes as per section 2.3.1. The protocol described in section 2.3.2 was then followed.

2.3.2 FACS

The sample was washed in HBSS, centrifuged at 300 rcf for 5 mins at 4ºC with the brake off and subsequently resuspended in 100µl of the primary antibody solution (rabbit anti-DDX4 antibody (ab13840; Abcam) at 1:10 concentration in blocking solution). After 20 mins incubation at 4ºC, two washing steps were performed with HBSS. The secondary antibody only control was washed once with HBSS and then both cell suspensions were resuspended in 250µl secondary antibody (goat anti-rabbit IgG antibody conjugated to allophycocyanin (APC) (cat no. 111-136-144; Jackson ImmunoResearch) at 1:250 concentration in blocking solution). Again, after 20 mins incubation at 4ºC, the cell suspensions were washed twice in HBSS. The negative control was washed once with HBSS and then all three cell suspensions were resuspended in 500µl of buffer solution (1% (v/v) FBS in HBSS) and subjected to FACS within 30 mins.

A BD FACSAria^{TM} II cytometer (BD Biosciences) was used for all experiments. A schematic of the method is depicted in Fig. 2.1.

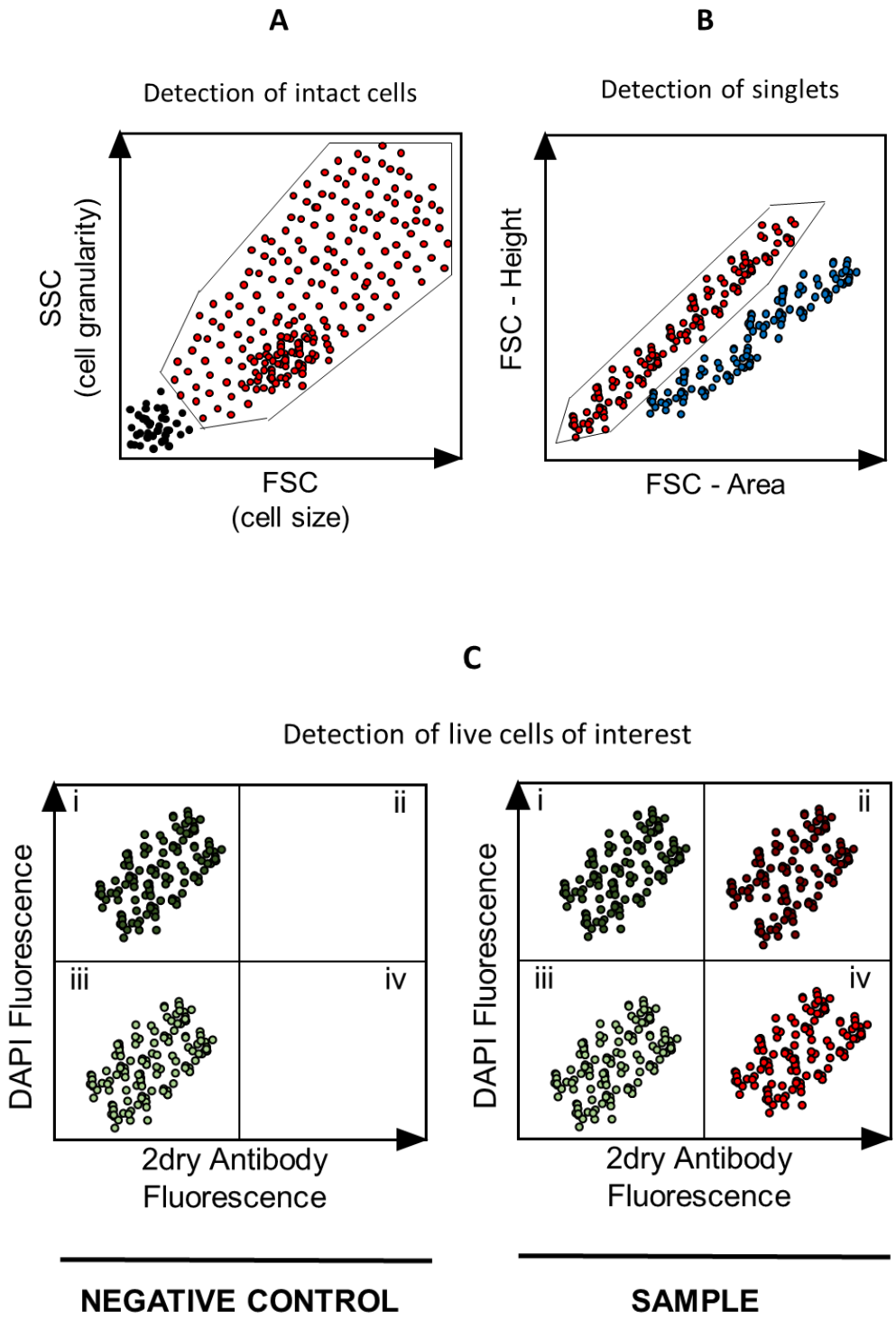
Figure 2.1. The basic principle underlying FACS. (A) To select a specific cell population a primary antibody against a cell surface protein is used. An appropriate secondary antibody with a fluorescent tag is then utilised to allow detection of the desired cell population by flow cytometry. Our experiments used a rabbit anti-DDX4 primary antibody and goat anti-rabbit APC-conjugated secondary antibody. **(B)** A schematic of the FACS process. A heterogenous sample of cells is placed in the cell sorter and single cells are subjected to a laser beam. Using the laser signal, the machine detects both the relative size of the cell (forward scatter; FSC) and granularity of the cell (side scatter; SSC). Fluorescence of several wavelengths is also detected and the cell sorter can be programmed to separate the cell suspension according to presence or absence of the desired fluorescence. (Red star = desired fluorescently tagged cells.)


A
Fluorescent Tag
Secondary Antibody
Primary Antibody
DDX4
OSC
B
Cell
Fluorescence/
Granularity
Detector
SSC
Cell Size
Detector
FSC
Mixed cell
population
Laser
Electromagnets
Positive
Sample
Negative
Sample

The cytometer located at the Queen's Medical Research Institute (QMRI) Flow Cytometry Facility (University of Edinburgh, UK) was used for initial experiments, whilst the cytometer located at the School of Biological Sciences (SBS) Flow Cytometry Core Facility (University of Edinburgh, UK) was used for experiments utilising the protocol in section 2.3.1.1. Staff at the Facilities (Shonna Johnston, Fiona Rossi and Will Ramsay at QMRI and Dr. Martin Waterfall at SBS) performed the analysis and cell sorting in discussion with myself. The cell sample was compared to the negative control sample and secondary antibody only sample and gates were set up in order to select APC-positive (fluorescence wavelength: 670nm), and therefore DDX4-positive populations (Fig. 2.2). A DNA stain, 4',6-diamidino-2-phenylindol (DAPI; 5µg/ml stock used at 1:200; fluorescence wavelength: 450nm; Life Technologies), was added before sorting. DAPI was used at a concentration (advised by FACS staff) at which live cells did not become stained, allowing dead cells (i.e. cells with damaged cell membranes) to be identified and thus excluded. Live DDX4-positive (i.e. APC-positive and DAPI-negative) cells that were to be cultured were collected directly into 1ml of OSC culture medium (detailed below in section 2.4) in 1 well of a 24 well plate (Corning) and immediately placed in an incubator at 37ºC / 5% CO_2. DDX4-positive cells collected for molecular characterisation of mRNA were collected in 350µl of RLT buffer (Qiagen) and 1% (v/v) β-mercaptoethanol (β-ME; Sigma-Aldrich) was subsequently added in a laminar flow hood. The cells were then processed as per section 2.9.

Figure 2.2. Simplified schematic of FACS plots to demonstrate how a specific cell population is selected. (A) "Events" (i.e. anything detected by the FACS machine, including cells and debris) are plotted according to their size and granularity. Debris (black dots) are excluded by their small size and intact cells (red dots) are then gated for (thin black line). **(B)** The intact cells are then examined and only single cells (red dots) are gated for (thin black line). Singlets are found in a diagonal line of approximately $x = y$, whilst doublets or aggregates of cells (blue dots) are found elsewhere on the plot. **(C)** Using fluorescence wavelengths specific to the secondary antibody and DAPI, live, single cells of interest can be detected. Comparisons with a negative control allows gates (thin black lines) to be applied so that secondary-antibody bound cells in the sample (quadrants **(ii)** and **(iv)** can be distinguished from

unbound cells (quadrants **(i)** and **(iii)**. As DAPI stains dead cells, cells in quadrant **(iv)** will be both live and positive for the protein of interest (in the case of this thesis, DDX4) and therefore will be collected.



2.4 In vitro Culture of Putative OSCs

Cells collected from FACS were cultured *in vitro* in OSC culture medium comprising MEMα (containing GlutaMAX^{TM}; Life Technologies) supplemented with 10% (v/v) FBS, 1 mM nonessential amino acids (NEAA), 1X-concentrated penicillin-streptomycin-glutamine (PSG; all Life Technologies), 1 mM sodium pyruvate, 1X-concentrated N-2 supplement (R&D Systems), 10^3 units/ml leukaemia inhibitory factor (LIF; Merck Millipore), 10 ng/ml recombinant human EGF, 1 ng/ml bFGF (both from Life Technologies), and 40 ng/ml glial cell-derived neurotropic factor (GDNF; R&D Systems) as per White *et al*. (White *et al.*, 2012). The culture medium was filter-sterilised through a 0.2 µm filtration device prior to use. Cells were cultured on plastic without the need for a feeder layer and fed on alternate days with 50-150µl of fresh OSC culture medium, depending on the size of the well the cells were in. Passaging of cells was performed once confluence was reached using 0.25% (v/v) trypsin-ethylenediaminetetraacetic acid (EDTA; Life Technologies).

2.4.1 Cell cryopreservation and thawing

DDX4-positive cells were cryopreserved using a method previously described for embryonic stem cells (Kent, 2009). Cells were trypsinised, centrifuged at 800 x g for 5 mins at room temperature and the cell pellet was gently resuspended in 500µl of OSC medium. Five hundred microlitres of cryopreservation medium (60% (v/v) FBS, 20% (v/v) OSC culture medium, 20% (v/v) DMSO) was then added drop-wise and gently mixed. The cell suspension was then transferred to a 2ml cryovial (Greiner Bio-One) and placed in an isopropanolol freezing container (Mr Frosty Freezing Container; Thermo Scientific). The following day the cryovial was placed in a liquid nitrogen storage container.

Cells were successfully re-established in culture following thawing. The cryovial was removed from liquid nitrogen and placed in a 37ºC water bath. The thawed contents were then transferred to a 15ml tube and 4ml of OSC culture medium was added. After centrifugation at 800 x g for 5 mins, the cell pellet was resuspended in fresh OSC culture medium and placed in 1 well of a 24 well plate.

2.5 Cell labelling with fluorescent markers

In order to track cells in *neo*-oogenesis experiments in Chapter 5, cells were labelled with fluorescent markers using two different methods.

2.5.1 Lentivirus Transduction

Cells were transduced to express green or red fluorescence using GFP- or mCherry-tagged lentiviruses (Lv-cppt-SFFV-IRES-hrGFP-opre at 1.6 x 10^7 viral particles/ml and Lv-cppt-SFFV-IRES-mCherry-opre at 1.2 x 10^6 viral particles/ml; designed and provided by Dr. Pamela Brown at the Biomedical Core Facility, Shared University Research Facilities (SuRF), University of Edinburgh, UK; Fig. 2.3). Both lentiviruses contained a central polypurine tract (cppt) to allow viral RNA integration and an optimised post-translational enhancer (opre) and internal ribosome entry site (IRES) to increase translation efficiency. A spleen focus-forming virus (SFFV) promoter, which aids stable transduction of stem cells, was used to drive fluorescence expression.

Both bovine and human cells were counted prior to transfection using a haemocytometer. In order to do this, cells were trypsinised, centrifuged at 800 x g for 5 mins and resuspended in OSC medium. Ten microlitres of the cell suspension was placed into the haemocytometer. The total number of cells in each of the four 4x4 grids were counted, with cells touching the left and bottom sides of each square included, but those touching the right and top sides excluded from the count. The total number was divided by four to obtain an average and this number represented the number of cells multiplied by 10^4 per ml of sample. The total number of cells in the suspension was then calculated depending on the size of the sample. Cells were plated down to achieve approximately 50% confluence the following day at which point they were exposed to OSC culture medium containing varying ratios of viral particles to cells (5:1, 10:1 or 20:1 for GFP experiments and 1:1, 5:1, 10:1 for mCherry experiments). Polybrene (hexadimethrine bromide; H9268, Sigma-Aldrich) was added to the viral medium at a concentration of 6 µg/ml to improve transduction efficiency. Polybrene is a positively-charged polymer which neutralises the repulsion between the viral particles and the negatively-charged sialic acid on the cell membrane. Viral medium was replaced with fresh medium after 24 (GFP) or 72

(mCherry) hours as this allowed adequate uptake of the lentivirus as assessed by microscopic visualisation. Fluorescing cells were analysed and imaged using an inverted microscope (Axiovert 200; Zeiss) and an attached Hamamatsu camera (Hamamatsu Photonics UK Ltd.). Images were compared to non-transfected cells to remove background or auto-fluorescence.

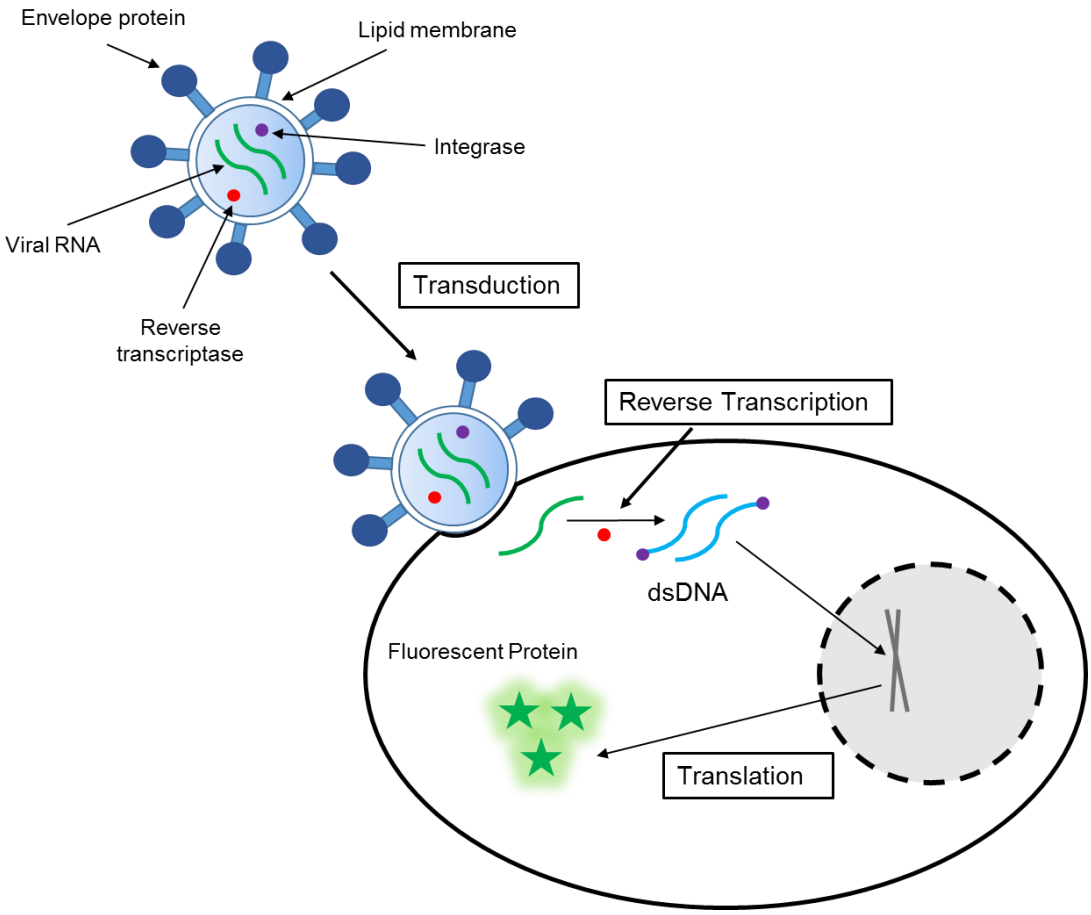


Figure 2.3. Lentiviral transduction of cells. Lentiviruses contain all the necessary components to allow integration of its RNA into the host cell genome, including reverse transcriptase and integrase. Lentiviruses transduce cells by cell membrane fusion with the target cell. The viral mRNA is then reverse transcribed to create double-stranded DNA, which is then integrated into the host DNA within the nucleus. The host cell then transcribes the integrated DNA and produces the desired protein, in this case a fluorescent protein (either GFP or mCherry).

Cells exposed to lentivirus were purified using FACS for GFP or mCherry fluorescence. Cells were trypsinised, resuspended in OSC medium and transported to a BD FACSAriaTM II cytometer (BD Biosciences; QMRI, University of Edinburgh) on fresh ice. Staff at the Facility (Shonna Johnston, Fiona Rossi and Will Ramsay) performed the analysis and cell sorting in discussion with myself. Transduced cell samples were compared to cells which had not been exposed to lentivirus and gates were set up using fluorescence wavelengths of 525nm and 582nm in order to select GFP- or mCherry-expressing cells, respectively. DAPI was added to exclude dead cells (fluorescence wavelength 450nm). Purified samples of live transduced cells were collected into OSC medium and plated down into 24 well plates. In order to analyse the long term stability of transfection, repeat flow cytometry was performed 2 weeks and 14 weeks (GFP-expressing bovine cells only) after purification, with gates applied using a sample of non-transfected cells as before.

2.5.2 Rhodamine Dextrans Labelling

Bovine and human cells were labelled with rhodamine-conjugated dextrans (Rhodamine B (570/590), D1824; Molecular Probes^{TM}, Thermo Scientific) to produce a red fluorescence (Fig. 2.4). Cells were exposed to 75µg/ml of rhodamine-conjugated dextrans in OSC medium for 24 hours before the medium was replaced with fresh OSC medium. As with the transfected cells, cells that endocytosed rhodamine-conjugated dextrans were analysed and imaged using an inverted microscope (Axiovert 200; Zeiss) and Zen software (Zeiss). They were compared to cells that had not been exposed to dextrans to remove background or auto-fluorescence (fluorescent wavelength: 561nm). No subsequent purification processes were performed.

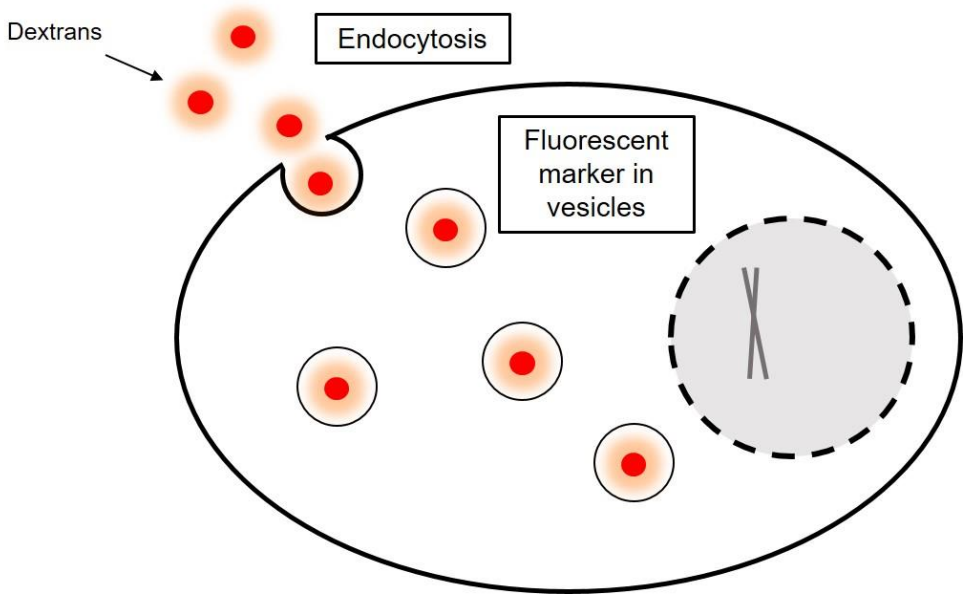


Figure 2.4. Tagging of cells with dextran conjugates. Dextrans in the surrounding culture medium are endocytosed by the cell and subsequently contained within vesicles in the cytoplasm. This confers a punctate fluorescence to the cell.

2.6 Fixing and Processing of Tissue

2.6.1 Fixation, embedding and sectioning

For immunohistochemistry (IHC) and immunofluorescence (IF) experiments, tissue was fixed in either 4% NBF for 24 hours or Bouin's solution for 3 hours before being transferred to 70% (v/v) ethanol. The tissue was either mechanically processed into paraffin wax using a standard protocol and a Histostar Embedding Workstation (Leica) by staff at SuRF (University of Edinburgh) or manually embedded. Tissue that was manually embedded was dehydrated by placing it in increasing concentrations of ethanol (70%, 90% and 100%) for 1 hour before being placed in cedarwood oil (VWR International) for 24 hours. The pieces were subsequently placed in toluene (Fisher Scientific) for 30 mins to remove the oil and then embedded in paraffin for 4 hours at 60ºC with hourly wax changes. A Leica RM2125 microtome (Leica) was used to cut paraffin-embedded tissue into 6µm sections and mounted onto electrostatically-

charged slides (Leica) using a 37ºC water bath. Slides were dried overnight at 50ºC and stored long-term at room temperature.

2.6.2 Dewaxing and rehydration

Tissue to be analysed by IHC and IF was dewaxed by placing slides in xylene (VWR International) for two washes of 5 mins each. Rehydration was then performed by placing the slides in decreasing amounts of ethanol (two washes in 100%, then 95%, 80% and 70%) for 20 secs each. Slides were then washed in tap water prior to either staining or antigen retrieval.

2.6.3 Haematoxylin and eosin staining

After dewaxing and rehydration, slides were placed in Harris haematoxylin solution (CellPath) for 2 mins to stain nuclei purple. The slides were then washed in tap water before being dipped in acid alcohol to remove non-specific staining. After a further wash in tap water, the slides were placed in Scott's Tap Water Substitute (STWS; CellPath) for 20 secs to increase the intensity of the haematoxylin stain and placed back in tap water. Slides were dipped briefly in 1% eosin (CellPath) to stain cytoplasm pink, washed in tap water and inspected under a light microscope to assess the quality of staining. Slides were then dehydrated through increasing concentrations of ethanol (70%, 85%, 90% and two washes of 100%) for 20 secs each, before being placed in xylene for 2 washes of 5 mins each. Slides were subsequently mounted with glass coverslips in a laminar flow hood using DPX mountant (Sigma-Aldrich) and left to dry at room temperature.

2.6.4 Immunohistochemistry

Immunohistochemistry was performed to identify the location of proteins of interest within tissue. Two different methods were utilised and their use will be specified in individual chapters. Specific primary and secondary antibody details will also be detailed in individual chapters.

2.6.4.1 Avidin/Biotin Peroxidase Detection

Slides were dewaxed and rehydrated as per section 2.6.2 before undergoing antigen retrieval in order to expose proteins that may have been masked during processing. Slides were heated in 0.01M citrate buffer (pH 6.0) using a microwave (5 mins at high power until boiling, then 15 mins on simmer setting, with regular replenishment of buffer). In order to block endogenous peroxidases and thus prevent non-specific binding by the primary and secondary antibodies, slides were subsequently incubated for 30 mins in 3% hydrogen peroxide (H_2O_2; Sigma-Aldrich) in methanol (VWR International). After repeated washing in 1x tris-buffered saline (TBS) containing 0.1% (v/v) Tween® 20 (TBST; both Sigma-Aldrich), non-specific binding by the secondary antibody was additionally ensured by blocking slides in species-appropriate normal serum (Vectastain® ABC Kit, Vector Laboratories) diluted in TBST (3 drops of serum in 10mls TBST). Two washes in TBST were again performed and the slides were incubated overnight with a suitable primary antibody (diluted in serum blocking solution) at 4ºC. For negative controls, the primary antibody was omitted and the slides were incubated in serum alone. The next day, the slides were washed in TBST twice, and incubated in the appropriate biotinylated secondary antibody (1 drop in 10mls TBST; Vectastain® ABC Kit) for 1 hour at room temperature. After two more washes in TBST, slides were incubated in avidin/biotin ABC complex (made up as per the manufacturer's instructions; Vectastain® ABC Kit) for 30 mins at room temperature and washed once more in TBST. A 3,3'-Diaminobenzidine stain (DAB; made up as per the manufacturer's instructions; Vector Laboratories) was used to visualise the bound antibody complexes and the slides were placed in tap water to stop the reaction when required. The sections were then counterstained with haematoxylin, treated with acid alcohol and STWS and finally dehydrated and mounted as per section 2.6.2.

2.6.4.2 ImmPRESS™ Peroxidase Detection

After dewaxing and rehydrating as described in section 2.6.2, antigen retrieval was performed in 0.01M sodium citrate (pH 6) in an Instant Pot® slow cooker (Instant Pot Company). The slide container was placed in 1.5 litres of deionised water in the cooker

and heated for 5 mins at full power before the cooker was allowed to vent steam for 20 mins with the slides inside. Endogenous peroxidases were blocked for 10 mins in Dako REAL™ peroxidase-blocking solution (Dako) and washed in 1x TBS several times. Slides were then incubated in 2.5% (v/v) normal horse serum (ImmPRESS™ kit; Vector Laboratories) for 30 mins at room temperature, washed in TBS twice and subsequently incubated in the appropriate primary antibody (diluted in serum blocking solution) overnight at 4ºC. The primary antibody was omitted for negative controls. The following day, slides were washed twice in TBS, and incubated with ImmPRESS™ reagent (Vector Laboratories) for 30 mins at room temperature. After further washing in TBS, antibody binding was visualised using DAB (Dako) and then slides were counterstained with haematoxylin, treated with acid alcohol and STWS and dehydrated and mounted as per section 2.6.2.

2.6.5 Immunofluorescence

IF was performed to identify the location of proteins of interest within tissue. As IF allows for more sensitive detection of proteins, lower antibody concentrations were used than for IHC. Specific primary and secondary antibody details will be detailed in individual chapters.

Slides were dewaxed and dehydrated as described in section 2.6.2 and subsequent antigen retrieval was performed using the Instant Pot slow cooker as per section 2.6.4.2. Endogenous peroxidases were blocked with Dako REAL™ peroxidase block (Dako) for 10 mins at room temperature and slides were then thoroughly washed in 1x phosphate-buffered saline (PBS; Life Technologies). Slides were subsequently blocked for 30 mins in species-appropriate serum blocking solution containing 20% (v/v) normal serum and 5% (w/v) BSA in 1x PBS. After further washing in PBS, slides were incubated in the appropriate primary antibody (diluted in blocking solution) overnight at 4ºC. For negative controls, the primary antibody was omitted. On the second day, two washes in PBS were performed and then an appropriate secondary antibody (diluted in blocking solution) was used, with an incubation of 30 mins at room temperature. After further washing in PBS, a fluorescein tyramide signal amplification detection kit (Perkin Elmer) was used at a concentration of fluorescein to amplification diluent of 1:50. Slides were incubated at room temperature for 10

mins and slides were exposed to as little light as possible during all subsequent steps. Slides were then washed twice in PBS and either DAPI (blue; Sigma-Aldrich), propidium iodide (PI, red; Sigma-Aldrich) or Sytox Green (green; Invitrogen™, Themo Scientific) nuclear counterstain was applied for 10 mins at room temperature (all at 1:1000, diluted in PBS). After further washing in PBS, slides were mounted with PermaFluor™ (Fisher Scientific) and stored at 4°C in the dark.

2.7 Fixing and Processing of Cells

2.7.1 Cell culture and Fixation

Cells to be analysed by immunocytochemistry (ICC) were cultured until confluent in 4- or 8-well Nunc Lab-Tek™ II chamber slides (Fisher Scientific). For bovine experiments, chamber slides had to be pre-coated with 0.01% (w/v) poly-L-lysine (Sigma-Aldrich) so that the cells remained adherent to the slide during processing. The slide chambers were filled with poly-L-lysine for 5 mins, then washed with distilled water (dH_2O) and air dried for 2 hours in a laminar flow hood prior to cells being plated down. Once the cells were confluent, the chamber slides were washed twice in 1x PBS and fixed in either Bouin's solution, 4% NBF or 50:50 methanol:ethanol for 10 mins, depending on the species of cells or primary antibodies used (further details in individual chapters). If methanol:ethanol fix was used, then the chamber slides were placed at -20ºC during this time. After further washing with PBS, fresh PBS was applied to each well, the slides were sealed with Parafilm (Bemis) and they were stored at 4°C until ICC experiments were performed.

2.7.2 Immunocytochemistry

ICC was performed to identify the expression and location of proteins of interest within cultured cells. It also allowed for comparisons in intensity of staining in experiments involving pharmacological treatments in Chapter 6.

If Bouin's or NBF was used as a fixative, then cells had to be permeabilised prior to proceeding with immunocytochemistry. Simultaneous permeabilisation and blocking was performed for 20 mins at room temperature using a permeabilisation and blocking

buffer: 1x PBS containing 0.2% (v/v) Tween® 20, 1% (w/v) BSA and 10% (v/v) species-appropriate normal serum. This was followed by a 10 min incubation in species-appropriate serum blocking solution comprising 5% (w/v) BSA and 20% normal serum in 1x PBS. As the methanol:ethanol solution both fixes and permeabilises cells, no further permeabilisation step was required for these cells. Cells fixed by this method were therefore washed in PBS twice and then blocked for 20 mins at room temperature in species-appropriate serum blocking solution comprising 5% (w/v) BSA and 20% (v/v) normal serum in 1x PBS. All slides, regardless of fixative, were washed in PBS twice more, then incubated with the required primary antibody diluted in the blocking solution above and stored at 4°C overnight. The primary antibody was replaced by serum blocking solution for negative controls. The following day, slides were washed in PBS and incubated in the relevant peroxidase-labelled secondary antibody diluted in blocking solution for 30 mins at room temperature. Following further washing in PBS, the chambers were removed from the slide. Fluorescein tyramide signal amplification was then performed as per section 2.6.5, with an incubation of 10 mins at room temperature and slides kept in the dark as much as possible thereafter. After further washing with PBS, a DAPI, PI or Sytox Green nuclear counterstain (all at 1:1000, diluted in PBS) was applied for 10 mins at room temperature. Slides were once again washed twice in PBS and mounted with PermaFluor™, before being stored at 4°C in the dark until ready for analysis.

2.8 Analysis of Histological Results

2.8.1 Light Microscopy

Tissue used in IHC experiments was examined for expression of proteins of interest under a Provis AX70 light microscope (Olympus). Images of slides were taken using a camera fitted to this microscope (AxioCam HRc, Zeiss) and scale bars were applied using AxioVision or Zen software (both Zeiss). Microsoft Powerpoint (Microsoft) was used to compile images.

2.8.2 Fluorescent Microscopy

IF and ICC experiments were analysed using a confocal laser scanning microscope (Zeiss LSM 710 or 780). Blue (DAPI) fluorescence was visualised using a wavelength of 405nm, green fluorescence wavelength was 488nm and red fluorescence wavelength was 561nm. Identical laser settings were used within each experiment. Images were acquired using the attached camera, scale bars were applied using the Zen software (Zeiss) and images composed in Microsoft Powerpoint (Microsoft). Negative controls were analysed in every experiment to ensure background or auto-fluorescence was corrected for and accurate interpretations of the data were made.

2.9 Gene Expression Analysis

In order to characterise the cells, analysis for the expression of messenger RNA (mRNA) of putative OSC and oocyte-specific markers was performed.

2.9.1 RNA extraction

Cells to be analysed for mRNA expression were lysed in 350µl RLT Buffer (Qiagen) containing 1% (v/v) β-ME (Sigma-Aldrich) and homogenised using a Qiashredder (Qiagen) as per the manufacturer's instructions. Total RNA was subsequently extracted using a RNeasy Micro Kit (Qiagen) as per the manufacturer's instructions, with on-column DNase I digestion for 30 mins. Total RNA was eluted in RNase-free water (Qiagen) and total RNA concentrations were measured by spectrophotometry using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific). Samples were stored at -80°C.

For the purposes of positive controls, RNA was also extracted from fetal bovine (166 days gestation) and human (19^{+5} weeks gestation) ovaries. To assess the specificity of the primers, adult mouse skeletal muscle RNA was used (tissue retrieved by Kelsey Grieve in Prof. Anderson's group). The ovary and muscle tissue was homogenised in the lysis buffer described above in a 1.5ml microfuge tube (Eppendorf) and then centrifuged briefly. The supernatant was then used for RNA extraction as detailed above.

2.9.2 cDNA synthesis

The Maxima First Strand cDNA Synthesis Kit (Thermo Scientific) was used to perform reverse transcription of extracted RNA as per the manufacturer's instructions, with the amount of RNA used dependent on the experiment (denoted hereafter as RT+ samples). A PTC-100 Thermal Cycler (Bio-Rad) was used with the cycling conditions described in Table 2.2.

Table 2.2. First strand cDNA synthesis Thermocycler programme for Maxima Kit.

Step	Temperature (ºC)	Time (mins)
1 (Denaturation)	25	10
2 (Extension)	50	15
3 (Termination)	85	5

For the purpose of negative controls to allow identification of contamination in subsequent experiments, reactions were performed using the same amount of total RNA with the Reverse Transcriptase enzyme omitted (RT- samples). The cDNA was stored at -20ºC.

2.9.3 Reverse transcriptase-polymerase chain reaction (RT-PCR)

To detect the presence of particular transcripts, RT-PCR was performed using 1µl of RT+ or RT- cDNA reactions and MyTaq^{TM} HS Red Mix (Bioline) to a total reaction volume of 25µl (Table 2.3). Forward and reverse primers were reconstituted at 25µM concentration, and used at a final concentration of 0.5µM.

Table 2.3. RT-PCR reaction mix using MyTaq™ HS Red Mix.

Reagent	Amount (µl)	Source
MyTaq™ HS Red Mix	12.5	Bioline
25µM Forward Primer	0.5	Integrated DNA Technologies
25µM Reverse Primer	0.5	Integrated DNA Technologies
RNase-free H_2O	10.5	Qiagen
cDNA	1	

Primer sequences for genes of interest were designed using Primer Blast (National Center for Biotechnology Information, NCBI) or obtained from the literature and purchased from Integrated DNA Technologies (IDT) (Tables 2.4, 2.5 (bovine experiments) and 2.6 (human experiments)).

Table 2.4. Primer sequences for bovine putative OSC genes of interest in RT-PCR experiments. Aliases for genes are denoted beneath abbreviations that will be used in this thesis. A gene fragment template DNA was used as a positive control for some genes (further details in Chapter 4).

Gene and Aliases	Accession No.	Forward Primer (5'-3')	Reverse Primer (5'-3')	Product size (base pairs)	Template size (base pairs)
POU5F1 (POU class 5 transcription factor 1; octamer-binding transcription factor-4; *OCT4*)	NM_174580	CGAAGCTGGACAAGGAGAAG	AGAGAACCCCCAGGGTGAG	159	164
LIN28	NM_024674	GGCCGTGGAGTTCACCTTTA	GTGGCAGTTTGCACTCCTTG	194	165
PRDM1 (PR Domain Zinc Finger Protein-1; B-lymphocyte-induced maturation protein-1; *BLIMP1*)	NM_001192936.1	CAAATGCCAGACGTGCAACA	GTGCACAAACTGCGTGAACT	211	N/A
DPPA3 (Developmental Pluripotency-Associated Protein 3; *STELLA*)	NM_001111109.2	ATCCATCGATGACGCTTCCC	ACTGCCCAGTCAGTTGAGATG	143	N/A
IFITM3 (Interferon-induced transmembrane protein 3; *Fragilis*)	NM_001078141.2	CACATCCCAGCCCTTGTTCA	TGTTGAACAGGGACCACACG	185	165
DDX4 (DEAD Box Polypeptide 4; *VASA*; *MVH*)	NM_001007819	GAGGACGAGATTTGATGGCTTG	GCACTGAACATAAGGGTCTGACG	475	428
CKIT	NM_001166484	AACACAATGGGACGGTGGAG	AGGGTGTGAGCATGGATTTGT	112	N/A
ACTB (beta-actin)	NM_173979	CTCTTCCAGCCTTCCTTCCT	GGGCAGTGATCTCTTTCTGC	178	N/A

Table 2.5. Primer sequences for bovine oocyte-specific genes of interest in RT-PCR experiments. Aliases for genes are denoted beneath abbreviations that will be used in this thesis. * Genes used in freshly isolated cell experiments. ** Gene used in cultured cell experiments. A gene fragment template DNA was used as a positive control for some genes (further details in Chapter 4).

Gene and Aliases	Accession No.	Forward Primer (5'-3')	Reverse Primer (5'-3')	Product size (base pairs)	Template size (base pairs)
HDAC 6 * (histone deacetylase 6)	NM_001098960.1	ATCTCCACTCTCTGGCTGAAGGTG	GGTCCATCATCTGCTGGTCATAGAC	318	448
GDF9 * (growth differentiation factor 9)	NM_174681.2	TTTCACAGGTGGCATTCCCTCC	ACACAGGATGGTCTTGGCACTG	431	446
SYCP3 * (synaptonemal complex protein 3)	NM_001040588.2	AGGATGCCATTGAAGAGAAGACC	CCCACTGCTGGAACAAAGTCAG	288	446
ZP3 * (zona pellucida glycoprotein 3 (sperm receptor))	NM_173974.3	ACGGATGGCGTGGTTAGGTTTG	CGAGAACACTGTGGTCCTGAATGG	243	449
Aromatase * (cytochrome P450, family 19, subfamily A, polypeptide 1; *CYP19*, *CYP19P1*)	NM_174305.1	CCCTCCCCCAAATGGAAAATC	GAGTAACATCTTGAGTTGAGCCACG	363	449
NOBOX ** (Newborn Ovary Homeobox)	HQ589330.1	TGTGGGTTGTGGGGAACAAG	TGCAGGTATGGAAACTGGGG	170	N/A

Table 2.6. Primer sequences for human genes of interest in RT-PCR experiments. Aliases for genes are denoted beneath abbreviations that will be used in this thesis. Primers marked by a * were obtained from the literature (White *et al*., 2012).

Gene and Aliases	Accession No.	Forward Primer (5'-3')	Reverse Primer (5'-3')	Product size (base pairs)
POU5F1	NM_001173531	ACATCAAAGCTCTGCAGAAAGAAC	CTGAATACCTTCCCAAATAGAACCC	127
LIN28	NM_024674	CGGGCATCTGTAAGTGGTTC	CAGACCCTTGGCTGACTTCT	191
NANOG	NM_024865	GACAAGGTCCCGGTCAAGAA	TGCTATTCTTCGGCCAGTTGT	223
PRDM1 *	NM_001198	AAACATGACCGGCTACAAGACCCT	GGCACACCTTGCATTGGTATGGTT	332
DPPA3 *	NM_199286	AGCAGTCCTCAGGGAAATCGAAGA	TATGGCTGAAGTGGCTTGGTGTCT	276
IFITM3 *	NM_021034	ATGTCGTCTGGTCCCTGTTC	GGGATGACGATGAGCAGAAT	205
DDX4	NM_024415	AAGAGAGGCGGCTATCGAGATGGA	CGTTCACTTCCACTGCCACTTCTG	239
CKIT	NM_000222	AAGGACTTGAGGTTTATTCCT	CTGACGTTCATAATTGAAGTC	345
GDF9 (Growth differentiation factor 9)	NM_005260.4	TAGTCAGCTGAAGTGGGACA	ACGACAGGTGCACTTTGTAG	277
RPL32 (60S ribosomal protein L32)	NM_000994	CATCTCCTTCTCGGCATCA	AACCCTGTTGTCAATGCCTC	153

Samples were subjected to identical cycling conditions using a PTC-100 Thermal Cycler (Table 2.7).

Table 2.7. RT-PCR Thermocycler Programme for MyTaq™ reaction mix.

Step	Temperature (°C)	Time
1 Initial denaturation of DNA	95	1 min
2 Denaturation of DNA	95	15 secs
3 Annealing of Primers	60	30 secs
4 Extension Reaction	72	30 secs
Steps 2 - 4 repeated for 35 cycles		
5 Final Extension	72	10 mins

Samples were resolved through 2.5% (w/v) agarose gels (Bioline; in 1x Tris-acetate-EDTA (TAE)) with GelRed™ (Cambridge Bioscience) staining at 0.01% (v/v) used to visualise DNA bands. Gels were run at 100 - 150 volts and 1x TAE was used as a running buffer. DNA ladders (100 bp; Bioline) were used to identify approximate product sizes. Gels were analysed under UV light using either a Gene Flash UV transilluminator (Syngene) or U:Genius3 UV transilluminator (Syngene) and images were captured using the attached cameras. In freshly isolated putative bovine OSC experiments, gene fragment template DNA (designed by Dr. Yvonne Clarkson using MacVector7.2 and purchased from IDT) was used as a positive control (Tables 2.4 and 2.5). Bovine or human fetal ovary cDNA was used as a positive control for cultured cells from each species to validate the use of the primers. Mouse skeletal muscle was used as a negative control. Two other types of negative controls were used to detect

contamination and subjected to the same procedures: RT- samples and samples with cDNA substituted for RNase-free water. Two reference genes, whose use had been previously established in Prof. Anderson's laboratory, were used to verify the effectiveness of the RT-PCR reactions: *β-actin* (*ACTB*) in bovine experiments and Ribosomal Protein L32 (*RPL32*) in human experiments. *ACTB* was also used as a reference gene for mouse skeletal muscle (Table 2.8).

Table 2.8. Primer sequences for the mouse reference gene, *ACTB*.

Gene	Accession No.	Forward Primer (5'-3')	Reverse Primer (5'-3')	Product size (base pairs)
ACTB	NM_007393	AGAGCTATGAGCTGCCTGACG	TGTGTTGGCATAGAGGTCTTTACG	179

2.9.4 Quantitative RT-PCR

For quantitative analysis of specific gene expression, quantitative RT-PCR (qRT-PCR) was performed using 1/10 dilutions of cDNA, Brilliant III Ultra-Fast SYBR Green Master Mix (Agilent Technologies), and forward and reverse primers at a final concentration of 500 nM (Table 2.9), with a final reaction volume of 10µl. Samples were placed in 96- or 384-well plates (Life Technologies).

Table 2.9. qRT-PCR reaction mix.

Reagent	Amount (µl)	Source
SYBR Green	5	Agilent Technologies
1/50 dilution of Reference dye (in RNase-free H_2O)	0.15	Agilent Technologies
25µM Forward Primer	0.2	Integrated DNA Technologies
25µM Reverse Primer	0.2	Integrated DNA Technologies
RNase-free H_2O	2.45	Qiagen
cDNA	2	

The ABI7900HTFast system (Applied Biosystems, Thermo Scientific) with SDS2.4 software was utilised to analyse the quantification cycle (C_q) value of genes of interest for each sample. This is a measure of the number of cycles required for the machine to detect a signal: the lower the C_q value, the more highly expressed the gene is. Consistent cycling conditions were used for each gene (Table 2.10).

Table 2.10. qRT-PCR ABI7900HTFast cycling conditions.

Step	Temperature (°C)	Time
1 (Hot start to activate DNA Taq Polymerase in SYBR Green Master Mix)	95	3 min
2 (Denaturation and annealing)	95	5 secs
3 (Extension)	60	15 secs
Steps 2 - 3 repeated for 40 cycles		
4 (Standard dissociation curve)	95 60 95	15 secs 15 secs 15 secs

Duplicate RT+ reactions were performed for each sample per gene of interest to increase reliability and one RT- reaction for each sample per gene of interest was performed to detect contamination. Bovine or human fetal ovary cDNA was used as a positive control for each species to validate the use of the primers.

Primer sequences were designed using Primer Blast (NCBI) and purchased from Integrated DNA Technologies (Tables 2.11 and 2.12).

Table 2.11. Primer sequences for bovine genes of interest in germ cell model qRT-PCR experiments.

Gene	Accession No.	Forward Primer (5'-3')	Reverse Primer (5'-3')
MSX1 (Muscle segment homeobox gene 1)	NM_174798.2	GCTGCCTCCTGTCCTTACAC	GATCTGCCTCTCCTGCAAAG
MSX2 (Muscle segment homeobox gene 2)	NM_001079614.2	AATGGCTGCAAAACCTATGC	GGAAAGGGTAAGATGCACCA
ID1 (Inhibitor of DNA-binding protein 1)	NM_001097568.2	TAAGACATGAACGGCTGCTA	TAGTCGATGACGTGCTGGAG
SYCP3 (Synaptonemal complex protein 3)	NM_001040588.2	GCTGGAAAGATTTGGAGCTG	AGCTTCTGCCTTTGCTCTTG
STRA8 (Stimulated by retinoic acid 8)	XM_010804529.1	TGCCAGCATGTACTCCAGAA	TCCTCCTCAACATCCTTCCC
DAZL (Deleted in azoospermia-like protein)	NM_001190811	GAAGGCAAAATCATGCCAAACAC	CTTCTGCACATCCACGTCATTA
ACTB	NM_173979	CTCTTCCAGCCTTCCTTCCT	GGGCAGTGATCTCTTTCTGC

Table 2.12. Primer sequences for human genes of interest in germ cell model qRT-PCR experiments.

Gene	Accession No.	Forward Primer (5'-3')	Reverse Primer (5'-3')
MSX1	NC_000004.12	CTCCTCAAGCTGCCAGAAGAT	GCTTACGGTTCGTCTTGTGTT
MSX2	NC_000005.10	TCGGAAAATTCAGAAGATGGA	GAGGAGCTGGGATGTGGTAA
ID1	NC_000020.11	CTCTACGACATGAACGGCTGT	TGCTCACCTTGCGGTTCTG
SYCP3	NC_000012.12	CAACTCCAACTCCTTCCAGA	AGCCGTCTGTGGAAGATCAG
RARβ (Retinoic acid receptor beta)	NM_000965	ATGCTGGATTTGGTCCTCTG	TGCACCTTTAGCACTGATGC
DAZL (Deleted in azoospermia-like protein)	NM_001190811	GAAGGCAAAATCATGCCAAACAC	CTTCTGCACATCCACGTCATTA
RPL32	NM_000994	CATCTCCTTCTCGGCATCA	AACCCTGTTGTCAATGCCTC

2.9.4.1 Statistical Analysis of qRT-PCR data

The expression of each analysed gene was calculated relative to the expression of the reference genes *β-actin* (bovine samples) and *RPL32* (human samples). Normalised gene expression was calculated using the $\Delta\Delta C_q$ method (Livak and Schmittgen, 2001): for each sample, the average C_q value of the reference gene was calculated and subtracted from the average C_q value of the gene of interest to determine the ΔC_q value. A "control" value from the experiment (e.g. a vehicle sample in Chapter 6) was then selected and subtracted from each ΔC_q value to calculate the $\Delta\Delta C_q$ value. As C_q values are on a logarithmic base 2 scale, the fold change in gene expression was determined by calculating $2^{-\Delta\Delta C_q}$. Microsoft Excel (Microsoft) was used for these calculations. Subsequent statistical analyses was then performed using GraphPad Prism (GraphPad Software, Inc.). Calculated $2^{-\Delta\Delta C_q}$ values were logarithmically transformed and checked for normalcy using the Shapiro-Wilks normality test (or Kolmorogov-Smirnov test if the $n \leq 3$). If data were normal, then they were analysed with the parametric one-way analysis of variance (ANOVA) with a post-hoc Tukey test. If the data were not normal, then they were analysed with the non-parametric Kruskal-Wallis test with post-hoc Dunn's test. A p level of < 0.05 was considered significant.

2.10 Western Blotting

2.10.1 Protein extraction and measurement

Protein expression was analysed in cultured cells. Cells were cultured in 100mm culture dishes (Corning) until confluent and then washed with DPBS. Cell lysis was performed using radioimmunoprecipitation assay (RIPA) buffer supplemented with 1 tablet of cOmplete™ Mini protease inhibitor (Roche) and 1 tablet of PhosSTOP phosphatase inhibitor (Roche) for 10 mins on ice. Cells were removed from culture plates using a cell scraper and placed in a 1.5ml microfuge tube. Cell lysates were centrifuged at 14000 x g for 10 mins at 4°C and the supernatants were carefully removed and stored at -20°C. Protein extraction from fetal bovine and human ovary was also performed for the purposes of positive controls. Snapfrozen bovine ovary (166 days gestation) and freshly dissected human ovary (19^{+6} weeks gestation) were homogenised in RIPA lysis buffer plus supplements as detailed above and centrifuged

at 14000 x g for 10 mins at 4°C. The supernatant was then removed and stored at -20°C.

Protein concentrations were measured in a 96-well plate (Corning) using Bio-Rad Protein Assay Reagents (Bio-Rad) as per the manufacturer's instructions. Triplicates of each sample were analysed as quality control. After 15 mins incubation at room temperature, absorbance measurements were made using a Multiskan Ex Microplate Reader (Thermo Scientific) and samples were compared with BSA concentration standards (ranging from 0 µg/ml to 1000 µg/ml) that had been run on the same plate in order to calculate protein concentrations.

2.10.2 Separation and Transfer

Lysates were added to 4xSDS Sample Buffer (625mM Tris, 5% (v/v) glycerol, 2% (v/v) SDS, 0.0025% (v/v) bromophenol blue in dH_2O; all Sigma-Aldrich)) containing 15% (v/v) β-ME and denatured at 99ºC for 6 mins. Lysates were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) on Bio-Rad mini-protean Tris-glycine TGX gels, with between 10-30µg of lysates added per lane depending on the experiment. Tris/glycine/SDS x1 (25mM Tris, 192mM glycine, 0.1% (v/v) SDS at pH 8.3; Bio-Rad) was used as a running buffer and gels were run at 125v until the loading dye had reached the bottom of the gel. A pre-stained protein ladder was used to identify approximate product molecular weights (PageRuler^{TM} Plus; Thermo Scientific). Gels were washed in dH_2O twice for 5 mins before being washed in Fast Semi-dry Transfer Buffer (Thermo Scientific) for 10 mins. Meanwhile, Immobilon FL membrane (Merck Millipore) was cut to size and soaked in methanol for 30 secs, dH_2O for 1 min and Transfer Buffer for 15 mins. Two pieces of filter paper per gel were also simultaneously soaked in Transfer Buffer for 15 mins. Semi-dry transfer was then performed by sandwiching the gel and membrane between the two pieces of filter paper in a semi-dry blotter at 25V for 9 mins.

2.10.3 Serum blocking, antibody probing and detection

After transfer, the membranes were blocked in a 1:1 ratio of Rockland blocking buffer (Rockland) to 1x PBS containing 0.05% Tween® 20 (PBST) for 1 hour at room

temperature. The membranes were then incubated in the required dilutions of primary antibodies (in blocking buffer) overnight at 4ºC. The following day, the membranes were washed four times in PBST for 5 mins each and incubated with the appropriate Alexa Fluor 680 conjugated secondary antibody for 1 hour at room temperature. After two 5 min washes in PBST and two 5 min washes in 1x PBS, a Li-cor Classic infrared imaging system (Li-cor Biotechnology) was used to detect the fluorescent signals of protein bands. The membranes were re-probed for the reference gene ACTB with a mouse anti-β-actin primary antibody (used at 1:5000; A5441, Sigma-Aldrich) and a donkey anti-mouse IRDye 800 conjugated secondary antibody (used at 1:10000; Rockland), before being re-imaged on the Li-Cor imaging system.

2.11 Commonly Used Solutions

2.11.1 Citrate Buffer

820ml of 0.1M sodium citrate was combined with 90ml of 0.2M citric acid monohydrate in 900ml of dH_2O and altered to a pH of 6.0 with sodium hydroxide (all Sigma-Aldrich), before being made up to 1000ml with dH_2O.

2.11.2 PBS

To make 1x PBS, 1 PBS tablet (Sigma-Aldrich) was added to 200ml of dH_2O.

2.11.3 RIPA buffer

RIPA buffer was made by combining 25mM Tris.HCl (12.11g Tris base added to 80ml dH_2O and adjusted to pH 7.5 with hydrochloric acid), 150mM sodium chloride, 1% (v/v) Triton X-100, 0.1% (v/v) SDS and 0.05% (w/v) sodium deoxycholate (all Sigma-Aldrich) in dH_2O.

2.11.4 TAE

A stock of 50x TAE was made by adding 484g Tris base, 114.2g glacial acetic acid and 100ml 0.5M EDTA (all Sigma-Aldrich) to 2000ml of dH_2O and adjusting the

solution to a pH of 8.0 with hydrochloric acid (Sigma-Aldrich). TAE (x1) was used in experiments by diluting the stock in dH_2O.

2.11.5 TBS

A stock of 10x TBS was made by adding 121.1g Tris base and 170g NaCl (both Sigma-Aldrich) to 2000ml of dH_2O and adjusting the solution to a pH of 7.4 with hydrochloric acid. TBS x1 was used in experiments by diluting the stock in dH_2O.

Chapter 3

Isolation and Culture of Putative OSCs

3.1 Introduction

3.1.1 Isolation of putative OSCs

As discussed in section 1.2.4, a few groups have reported the isolation of OSCs in adult mice (Zhang *et al.*, 2008, Zou *et al.*, 2009, Pacchiarotti *et al.*, 2010, Zou *et al.*, 2011, Hu *et al.*, 2012, White *et al.*, 2012, Hernandez *et al.*, 2015, Xiong *et al.*, 2015, Lu *et al.*, 2016), rats (Zhou *et al.*, 2014), pigs (Bui *et al.*, 2014), rhesus macaque monkeys (Hernandez *et al.*, 2015) and humans (White *et al.*, 2012, Hernandez *et al.*, 2015). Three different methodologies have been used, ranging in complexity and each with their own benefits and disadvantages: from simple dissociation of ovarian cortex with analysis of cells that established in culture, to immunomagnetic cell selection and fluorescence-activated cell isolation (Table 3.1). Selection for both Ddx4 and Ifitm3 has been used successfully (Zou *et al.*, 2009, Zou *et al.*, 2011, White *et al.*, 2012, Hernandez *et al.*, 2015, Xiong *et al.*, 2015, Lu *et al.*, 2016), although it has been suggested that Ifitm3-based selection is more effective that Ddx4-based methods, with significantly more (almost twice as many) putative OSC cells isolated using Ifitm3 (Zou *et al.*, 2011). The authors postulated that this may be because Ifitm3 offers more binding sites to its antibody than the hypothesised transmembrane domain of Ddx4 (Zou *et al.*, 2011). Ifitm3 is a cell surface protein (Saitou *et al.*, 2002), thus its use also avoids some of the controversy around the use of Ddx4 (see section 1.2.5.2.).

Table 3.1. Some of the advantages and disadvantages to the three methods used for isolating putative OSCs to date.

Technique	Pros	Cons
Culture of dissociated cortex with no selection for protein expression	• Technically more straightforward • Rapid and cheap • Possibly less damaging to cells as subjected to fewer processing steps	• Results in impure cell populations (although can select out cells containing protein of interest if it is linked to a fluorescent marker) • Relies on population of interest outgrowing other cell populations
Immunomagnetic cell selection (magnetically activated cell sorting; MACS)	• Selects for cells expressing a desired protein of interest on the cell surface • Quicker than FACS • Cheaper than FACS	• No exclusion of dead cells • No selection on the basis of cell size (i.e. OSCs and damaged oocytes both collected) • Inadequate disaggregation is detrimental to sorting efficiency (Zou *et al.*, 2011)
FACS	• Selects for cells expressing a desired protein of interest (can be internally expressed if the protein is linked to a fluorescent marker) • Can select cells on basis of size, so can isolate a purer population than MACS • Can exclude dead cells	• Requires expensive equipment • Takes longer than MACS • Inadequate disaggregation is detrimental to sorting efficiency • Can cause more cell damage than MACS

3.1.2 *In vitro* culture of putative OSCs

Once isolated, the medium in which the stem cells are cultured is critical for healthy growth and for maintenance of an undifferentiated state in order for the cell line to propagate. With this in mind, all groups who have reported the *in vitro* culture of putative OSCs have used a serum-containing medium to provide growth factors, and all but one (Pacchiarotti *et al.*, 2010) supplemented the medium with a combination of specific growth factors, such as EGF, bFGF and LIF in order to both encourage proliferation and prevent differentiation (Zou *et al.*, 2009, Hu *et al.*, 2012, White *et al.*, 2012, Bui *et al.*, 2014, Zhou *et al.*, 2014, Hernandez *et al.*, 2015, Xiong *et al.*, 2015, Lu *et al.*, 2016). The inclusion of LIF in particular has proved beneficial, with Hu *et al*. reporting that cells in LIF-containing medium formed more colonies (Hu *et al.*, 2012). Despite the inclusion of factors in the culture medium that prevent differentiation of stem cells, nearly all groups have reported the spontaneous differentiation of some of the cells into OLCs during *in vitro* culture, which have been characterised on the basis of morphology and/or molecular analysis (Zou *et al.*, 2009, Pacchiarotti *et al.*, 2010, Hu *et al.*, 2012, White *et al.*, 2012, Bui *et al.*, 2014, Zhou *et al.*, 2014, Hernandez *et al.*, 2015). Moreover, to date, all groups have used a feeder layer to help the putative OSCs establish in culture, although White *et al*. have reported that cells will establish without such a layer, but at lower efficiency (Zou *et al.*, 2009, Pacchiarotti *et al.*, 2010, Hu *et al.*, 2012, White *et al.*, 2012, Bui *et al.*, 2014, Zhou *et al.*, 2014, Hernandez *et al.*, 2015, Xiong *et al.*, 2015, Lu *et al.*, 2016).

3.1.3 Fluorescent labelling of putative OSCs

The definitive test of a putative OSC is whether the OSC can form functional oocytes. The conditions used to demonstrate this differentiation thus far have required that the cells are traceable in order to ascertain whether observed oocytes are pre-existing or have been derived from OSCs. As such, cells have been successfully induced to express GFP by both viral transduction and liposome-mediated transfection and such cells have continued to propagate in culture (Zou *et al.*, 2009, Zhang *et al.*, 2011, White *et al.*, 2012, Wolff *et al.*, 2013, Wolff *et al.*, 2014, Zhou *et al.*, 2014, Xiong *et al.*, 2015, Lu *et al.*, 2016).

3.1.4 Aims of this chapter

The overall aim of the experiments detailed in this chapter was to attempt the recapitulation of putative OSC isolation and *in vitro* culture. The principal aim was to isolate putative OSCs from human ovarian cortex, in order that the resulting data were as clinically applicable as possible. However, we chose to undertake the experiments using bovine ovarian cortex initially, allowing the protocol to be optimised in our laboratory before testing it on human tissue. The reason for this was threefold: (1) bovine tissue is more easily accessible in large quantities than human tissue, which is much more precious, (2) the cow is a good large animal model for human reproductive physiology and therefore data from bovine tissue may be more translatable to humans than rodent data and (3) attempting the isolation of bovine OSCs was a novel experiment not previously reported in the literature. With regards the second point, both cows and humans are mono-ovular species, with similar follicular development (see Fig. 1.2) and as such cows are considered to be a valuable and relevant model for human ovarian development (Campbell *et al.*, 2003).

Due to a previous collaboration between Prof. Telfer and Jonathan Tilly and given that the method had already successfully isolated putative OSCs from human tissue, for the purposes of this research isolation was attempted using the FACS-based protocol his group had published (White *et al.*, 2012, Woods and Tilly, 2013). The fact that FACS potentially yields a purer isolated population than MACS and that our group had access to the required FACS equipment further supported the decision. The protocol utilises DDX4 for cell selection, which is the subject of great debate (see section 1.2.5.2); however, given that its use had been previously validated in mice (Zou *et al.*, 2009) and humans (White *et al.*, 2012), White *et al.*'s protocol was adhered to (White *et al.*, 2012, Woods and Tilly, 2013). The culture medium detailed by the Tilly group (White *et al.*, 2012, Woods and Tilly, 2013) would also be used if cells were isolated. Given that the function of any isolated cells was to be tested, a secondary aim of the research in this chapter was to fluorescently label isolated cells. Due to local expertise in lentiviral vectors, this was the proposed method to be used, in place of a retrovirus. At the time of the experiments within this chapter, this was a novel technique, which has subsequently been validated by other groups in this cell population (Wolff *et al.*, 2013, Wolff *et al.*, 2014, Xiong *et al.*, 2015, Lu *et al.*, 2016).

3.2 Materials and Methods

3.2.1 Immunohistochemistry of DDX4-positive cells in ovarian cortex

Immunohistochemistry was performed to identify DDX4-positive cells within adult ovarian cortex. For bovine experiments, a small piece of fresh cortex was dissected and fixed in 4% NBF for 24 hours as per section 2.1.1, then processed into paraffin and sectioned onto slides as per section 2.6.1. IHC was performed using ImmPRESS™ peroxidase detection as detailed in section 2.6.4.2, with a polyclonal rabbit anti-DDX4 primary antibody (used at 1:500; ab13840, Abcam, Cambridge, UK). The primary antibody was omitted for negative controls.

For human experiments, a biopsy of ovarian cortical tissue was taken with written and informed consent from a 22 year old woman undergoing an elective Caesarean section. A small piece was fixed in 4% NBF for 24 hours, processed into paraffin and sectioned onto slides as per section 2.6.1. IHC was performed by a colleague (Dr. Marie McLaughlin) using avidin/biotin detection as per section 2.6.4.1, using normal goat serum (Vectastain® ABC Kit, Vector Laboratories) as a blocking agent, the same polyclonal rabbit anti-DDX4 primary antibody (used at 1:500; ab13840, Abcam) and a biotinylated goat anti-rabbit IgG secondary antibody (Vectastain® ABC Kit, Vector Laboratories). The primary antibody was omitted for negative controls.

3.2.2 Isolation of putative OSCs

Putative OSCs were isolated from vitrified adult bovine and human ovarian cortex using the methods detailed in section 2.3. Due to low cell yields, cells were either collected for culture *or* for characterisation. Moreover, when cells were collected for culture, only one population could be collected at a time as they were collected directly into a well of a 24-well plate and the machine did not have the capabilities to sort two different populations simultaneously into a plate.

To estimate the size of the bovine cells, beads of known diameter (3.2µm, SPHERO™ Rainbow 6-peak Calibration Particles; Spherotech), 6µm (BD Calibrite™ Beads; BD Biosciences) and 10µm (Flow-Check™ Fluorospheres; Beckman Coulter) were

placed in the BD FACSAria™ II cytometer and their average FSC values compared with those of the isolated cell populations.

To attempt to investigate the ploidy of the bovine DDX4-positive cells, a FACS experiment was performed. Bovine cortex was thawed, dissociated and subjected to FACS as per section 2.3. The collected isolated DDX4-positive cells were then placed back in the BD FACSAria™ II cytometer and an attempt to measure the intensity of DAPI staining as an indication of ploidy was made.

One experiment was performed to examine whether IFITM3 could be used to select for putative OSCs. The methods detailed in section 2.3 were utilised, with the exception of the antibodies. An anti-IFITM3 primary antibody (ab74699; Abcam, used at 1:10) and a goat anti-rabbit secondary antibody conjugated to Alexa Fluor® 488 (ab150077; Abcam, used at 1:250) were used instead.

Cells isolated by the alternative dissociation protocol (section 2.3.1.1.) were collected for molecular analysis (section 2.3.2).

3.2.3 *In vitro* culture and cryopreservation of putative OSCs

Putative OSCs which established in *in vitro* culture were cultured, passaged and cryopreserved as per section 2.4.

In initial experiments, cells were plated onto mouse embryonic feeder cells (MEFs; VHBio Ltd.) as a feeder layer. Twenty-four well culture plates (Corning) were coated in 0.1% (w/v) gelatin for 3 hours at 37ºC before the gelatin was removed and the MEFs were added to the wells. MEFs, stored in liquid nitrogen, were thawed in a 37ºC water bath for 2 mins and resuspended in MEF culture medium as per the manufacturer's instructions. In brief, thawed MEF suspension was added at a 1:10 ratio to Dulbecco's Modified Eagle Medium (DMEM; Life Technologies) containing 10% (v/v) FBS and 1X-concentrated PSG, centrifuged at 270 x g for 5 mins and the supernatant discarded. The pellet was resuspended to a concentration of 1 x 10^5 MEFs/ml and 0.5ml of cell suspension was added to each well (i.e. 50,000 cells/well). The plates were incubated at 37ºC / 5% CO_2. The MEF-coated plates were made up at least 24 hours prior to putative OSCs being placed in the wells.

Cells from Patient 2 at passage 10 (P10) were used for live cell imaging purposes, with cells recorded by an inverted microscope (Axiovert 200; Zeiss) for 16 hours, with images taken every 30 mins.

3.2.4 Cell labelling with fluorescent markers

Cells were labelled with fluorescent markers by lentivirus transduction or rhodamine dextrans endocytosis as per section 2.5.

3.2.5 Statistical analyses

The estimated cell sizes of the bovine populations were calculated by comparing the average FSC value of the isolated populations to the average FSC of beads of known size. The FSC values of the beads were plotted on a scatterplot of cell size by mean FSC and a linear trend line was calculated using Microsoft Excel 2013. Using the equation, $y = mx + c$, from this trend line, the cell sizes of the isolated populations could be approximated by inputting their FSC values.

Growth rates of bovine and human cells were monitored by recording the required frequency and magnitude of passaging. Growth curves were then calculated using GraphPad Prism 5.0 (GraphPad Software, Inc.; California, USA). Data was logarithmically transformed by log base 2 to graphically represent the data as $y = mx + c$. GraphPad Prism 5.0 was then used to statistically analyse the resultant slopes (gradients) to assess if significant differences in growth rates existed. To compare the growth rates of the bovine cells and human cells, the mean slope ("m") and intercept ("c") values were calculated for the cell lines of each species and plotted graphically. To assess the statistical significance between the growth rates of the two species, the slope values ("m") for each species were compared by an unpaired, two-tailed t test, after testing for normalcy using the Kolmogorov-Smirnov test.

A dose response curve for GFP lentiviral transduction of bovine cells was calculated using GraphPad Prism 5.0 by logarithmically transforming the lentiviral particle: OSC ratio and plotting the values against transduction rate (%). A non-linear regression curve fit was performed using an exponential growth equation.

3.3 Results

3.3.1 DDX4-positive cell populations in adult ovarian cortex

Immunohistochemistry was performed on adult bovine and human ovary to determine the location of DDX4-expressing cells in adult ovarian cortex that are detected by the anti-DDX4 antibody used for putative OSC isolation. Two separate populations of DDX4-positive cells were identified in both species: (a) oocytes in follicles of varying stages of development and (b) a much smaller population, distributed sparsely and apparently randomly throughout the cortex, measuring 6 - 10 µm in diameter (Fig. 3.1). This latter population was rarely detected and had an expanded cytoplasm compared with surrounding stromal cells. Moreover, the cells did not appear to be associated with any particular structures, such as follicles, blood vessels, or the surface epithelium.

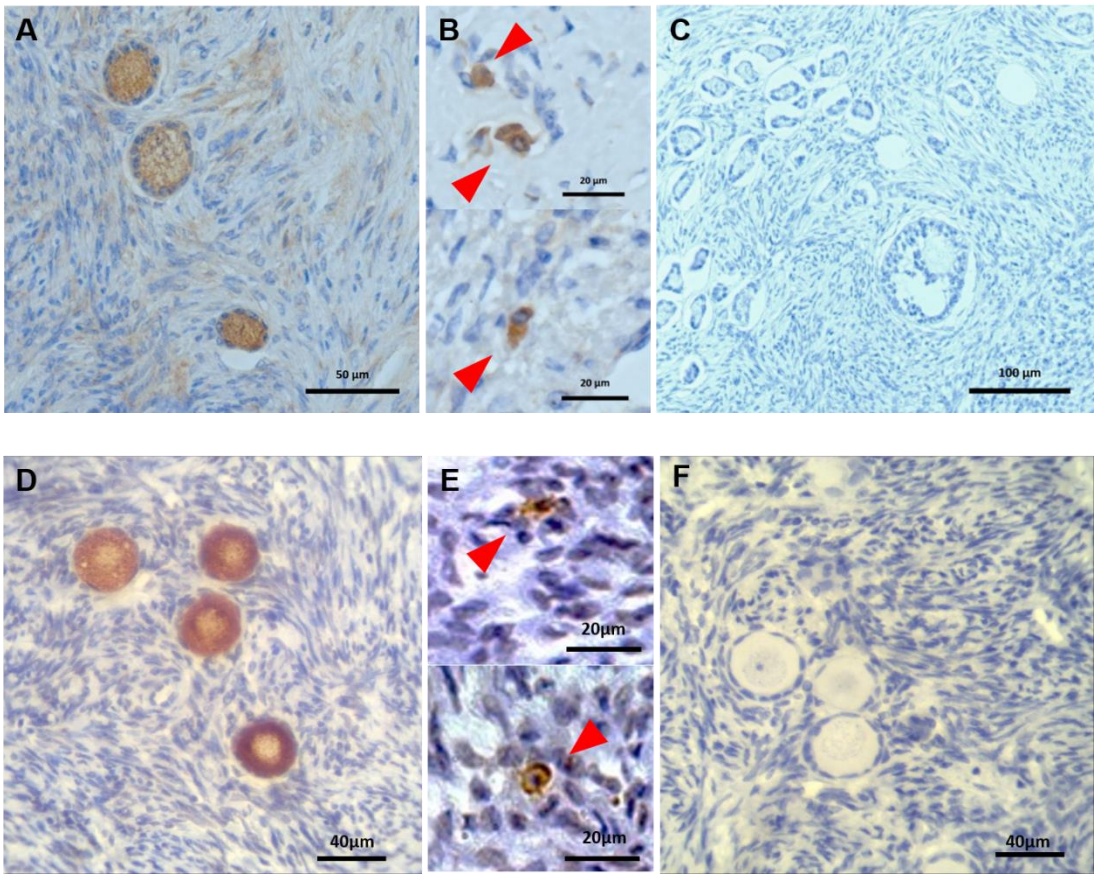


Figure 3.1. Immunohistochemical detection of DDX4-positive cells in adult bovine (A-C) and human (D-F) ovarian cortex indicated by brown staining. Two separate populations were identified: oocytes within follicles (**A** (scale bar = 50µm) and **D** (scale bar = 40µm)) and a smaller population not associated with follicles or other structures and measuring 6 - 10 µm in diameter ((**B**) and (**E**); red arrows; scale bar = 20µm). The primary antibody was omitted for negative controls (**C** (scale bar = 100µm) and **F** (scale bar = 40µm)).

3.3.2 Isolation and culture of bovine DDX4-positive cells

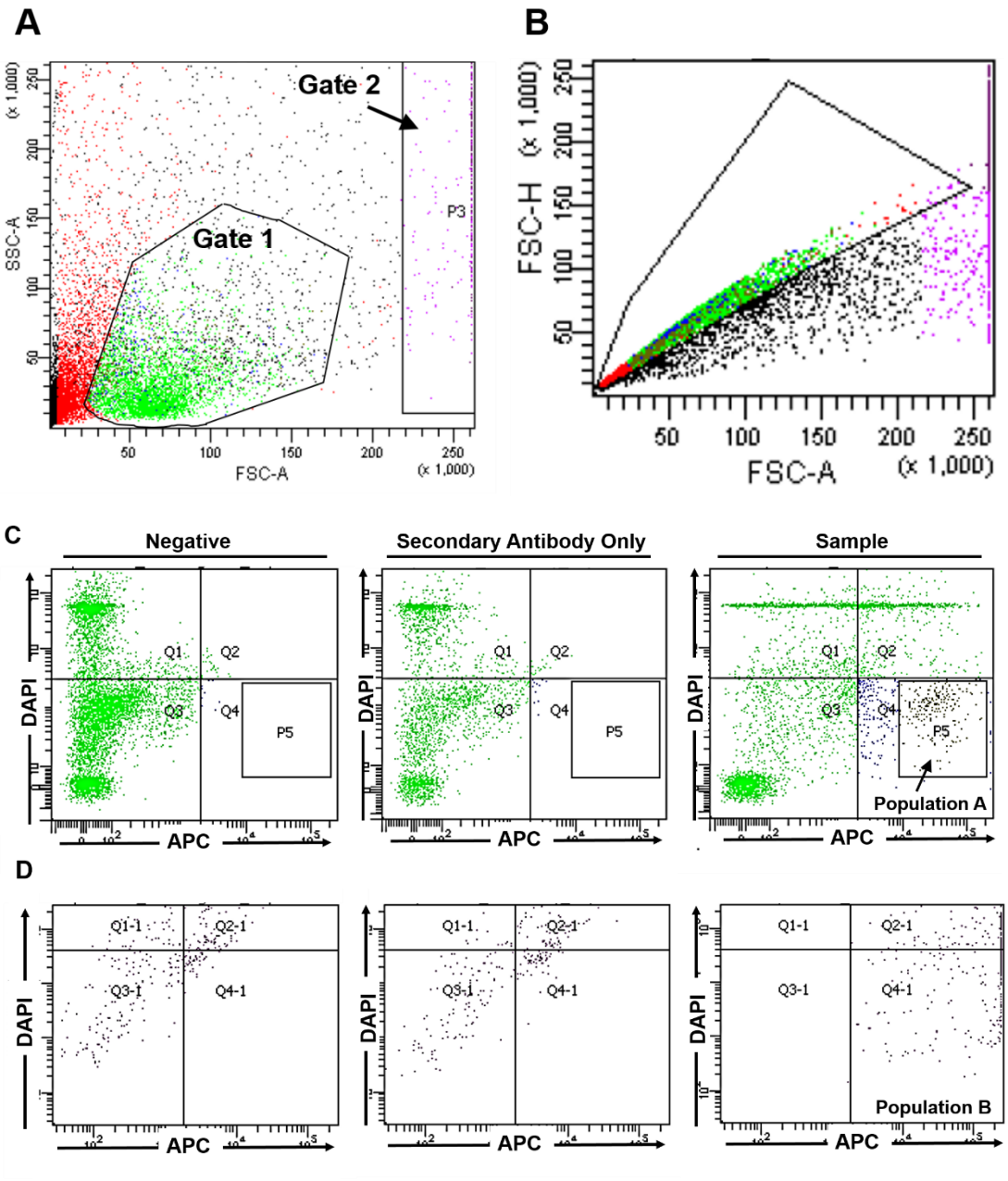
3.3.2.1 Cell isolation

The FACS-based protocol previously published (White *et al.*, 2012, Woods and Tilly, 2013) was optimised for use in bovine tissue initially over the course of 4 months, with adaptations to the enzymatic dissociation steps. A range of collagenase type IV concentrations was tested, with 600u/ml deemed optimal as this allowed adequate disaggregation compared with a lower concentration (400u/ml) and more acceptable levels of cell death as detected by DAPI during flow cytometry compared with a higher concentration (800u/ml). This was an increase in concentration from that previously published (400u/ml; White *et al.*, 2012, Woods and Tilly, 2013). The DNase I: collagenase ratio was also increased tenfold from that used by White *et al.* (White *et al.*, 2012, Woods and Tilly, 2013) from 1:1000 to 1:100 as this improved the cell yield during FACS. An additional alteration to the White *et al*. protocol (White *et al.*, 2012, Woods and Tilly, 2013) was the soaking of the filter in a serum-containing medium prior to use, which also enhanced cell yield as ascertained subjectively by the size of the cell pellet retrieved following filtration.

Varying methods of mechanical disaggregation were also trialled, including using a pestle and mortar, a cell dissociation sieve and a homogeniser, all of which were too harsh on the tissue and did not produce intact cells as observed under a light microscope. A gentleMACS^{TM} Dissociator was subsequently used and the protocol described by White *et al*. (White *et al.*, 2012, Woods and Tilly, 2013) for mechanical dissociation was followed for all experiments that yielded DDX4-positive cells.

Once the cell isolation protocol was adjusted, DDX4-positive cells were consistently isolated from bovine ovarian cortex (n = 9; Fig. 3.2). Two discrete populations were identified, with Population A containing cells smaller in cell size but higher in prevalence than Population B.

Figure 3.2. Representative FACS plots for dissociated bovine ovarian cortex. (A) All events detected by the machine were gated so that only intact cells were analysed. Gate 1 contained smaller cells than Gate 2. **(B)** Single cells were subsequently gated for and used for fluorescent analyses. **(C)** Population A cells were derived from Gate 1. No APC-positive cells (and therefore DDX4-positive cells) were detected if the cell suspension had no antibodies (negative) or the secondary antibody only. DAPI staining allowed for exclusion of dead cells such that live, DDX4-positive cells were found in the bottom right quadrant. The area of increased density of cells within that quadrant was gated as the DDX4-positive cells. **(D)** Population B cells were derived from Gate 2. Again, no APC-positive cells were detected in the two negative controls and live, DDX4-positive cells were located in the bottom right hand quadrant.


A
Gate 2
Gate 1
P3
SSC-A
(x 1,000)
FSC-A
(x 1,000)
B
FSC-H
(x 1,000)
FSC-A
(x 1,000)
C
Negative
Secondary Antibody Only
Sample
DAPI
APC
Q1
Q2
Q3
Q4
P5
Population A
D
Q1-1
Q2-1
Q3-1
Q4-1
Population B

These cell populations were scarce, making up a very small proportion of the dissociated ovarian cortex, with an average yield of 3.8% ± 1.2% (± S.E.M.) for Population A and 1.0% ± 0.2% (± S.E.M.) for Population B. This equated to raw cell numbers per isolation ranging from 171 - 1526 for Population A and 199 - 1033 for Population B (Table 3.2). By comparison with beads of known size, the sizes of the cells could be estimated, with the average size of Population A being 14 µm in diameter and the average size of Population B being 16 µm. To attempt to investigate the differences between the two populations, an experiment was performed on freshly isolated cells to analyse their ploidy using DAPI. Unfortunately, there were too few cells (Population A: 351 cells, Population B: 81 cells) to perform the experiment.

Table 3.2. Raw numbers of DDX4-positive cells isolated during each bovine experiment and the purpose of each experiment. Population A was smaller in cell size but more abundant than Population B. * Population B was not identified until Experiment 2, therefore none were collected in Experiment 1. ** No Population B cells were collected in Experiments 6 or 9 due to technical difficulties resulting in not enough sample remaining after Population A collection was performed.

Expt.	Pop. A (no. of cells)	Pop. B (no. of cells)	Utilisation of cells
1	718	*	Characterisation
2	277	1033	Culture (Cell Line 1)
3	1526	199	Characterisation
4	171	430	Culture (Cell Line 2)
5	454	261	Culture (Cell Line 3)
6	646	**	Characterisation
7	1338	667	Culture (Cell Line 4)
8	1000	318	Culture (Cell Line 5)
9	274	**	Culture (Cell Line 6)

An experiment was performed with an anti-IFITM3 antibody to assess whether a similar population could be detected using this method. No convincing IFITM3-positive population(s) could be identified, however, and due to time constraints this technique was not pursued further.

When the dissociation protocol was changed to the one devised by colleagues in Prof. Telfer's group, the cell yields increased. Initially only one population could be detected, with a yield of 14.4% ± 4.8% (S.E.M.) (raw numbers of 784 - 64872, n = 5; experiments performed by Dr. Marie McLaughlin and Dr. Yvonne Clarkson from Prof. Telfer's group). Once gating strategies were altered, the two populations were again found with yields of 19.4% ± 5.4% (S.E.M.) of total cell population for Population A and 5.6% ± 1.2% (S.E.M.) for Population B (n = 4; experiments performed by myself). This equated to raw cell numbers of 115355 - 200000 for Population A and 13030 - 140000 for Population B. These cells were all used for characterisation of freshly isolated cells (see Chapter 4) and were not cultured.

3.3.2.2 *In vitro* culture of isolated cells

In initial experiments, mouse embryonic feeder cells (MEFs) were used to encourage and support the growth of isolated cells subsequently added to the wells. This was performed in adherence to the White *et al*. methodology (White *et al.*, 2012, Woods and Tilly, 2013); however, no proliferating putative OSC colonies were observed and the MEFs did not adhere well to the plates, thus ascertaining discrete cell populations was not possible. The use of MEFs was therefore abandoned and isolated cells were clearly seen establishing in culture consequently. Population B cells established in culture following 6 individual experiments (Table 3.2). This was in contrast to Population A which only established once in culture (Expt. 9), although the growth and morphology of these cells once established were indistinguishable from Population B. Proliferating cells were first visible within 1 week of culture in colony-like clusters with ill-defined edges and were ready for passage 3 - 7 days later. After the first passage, cells proliferated evenly over the plate. The cells exhibited an elongated shape with protruding cytoplasmic branches (Fig. 3.3(a)). In accordance with other groups who have reported the isolation of OSCs, the cultured bovine cells

spontaneously produced floating spherical cells of 30-40 µm in diameter (Fig. 3.3(b)). This occurrence was very infrequent: as an estimate, at least one OLC and no more than ten OLCs were observed during the entire *in vitro* culture period of each cell line and only in the later passages (i.e. after passage 10).

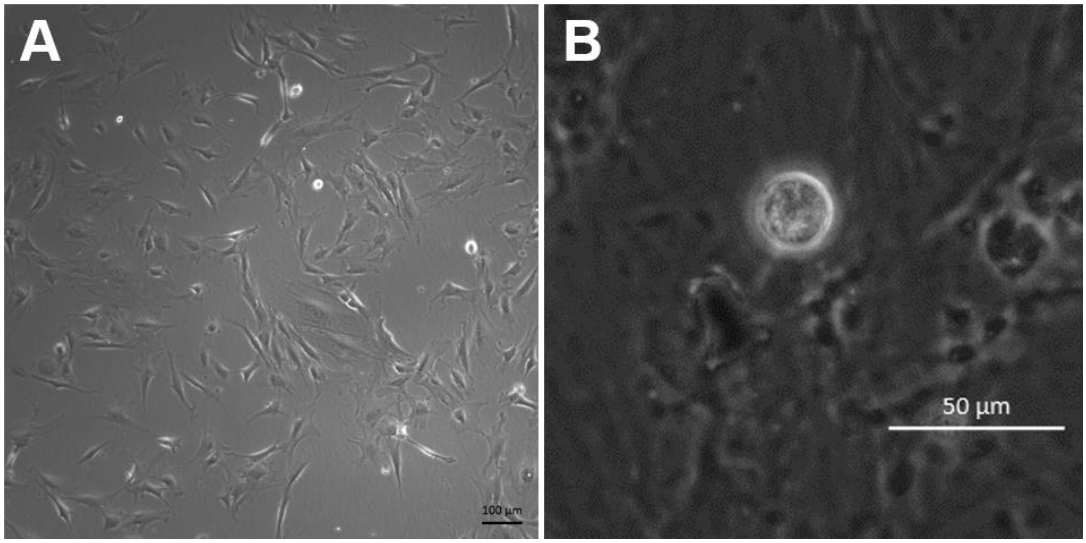


Figure 3.3. Bovine cells cultured *in vitro*. (A) Cultured bovine cells were elongated in shape. Their morphology was maintained during long-term culture (Cell Line 4, passage 17; scale bar = 100µm). **(B)** An example of a rarely observed oocyte-like cell (OLC) spontaneously formed during *in vitro* culture. Scale bar = 50µm.

Isolated bovine cells were propagated continuously for up to 6 months, with Cell Line 4 undergoing more than 30 passages. The different cell lines showed statistically significant differences in growth rates ($p = 0.005658$; Fig. 3.4).

Cultured cells could be cryopreserved for future use; however, successful thawing and reestablishment in culture of cryopreserved cells was only possible if 10% (v/v) DMSO was used as a cryoprotectant during the freezing process.

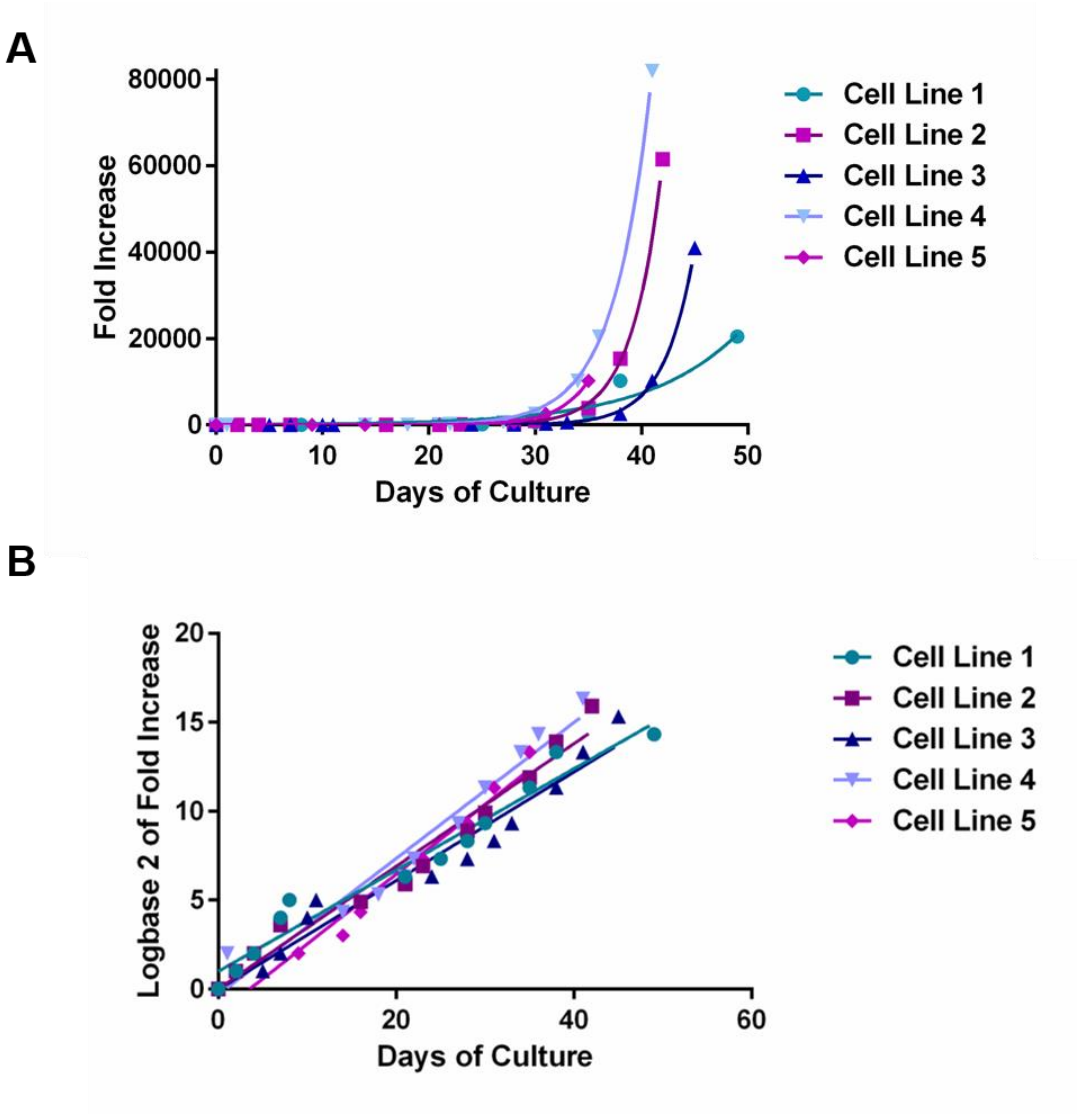


Figure 3.4. Bovine cell lines exhibited significantly different rates of growth during *in vitro* culture. **(A)** Non-transformed data demonstrating that Cell Line 4 grew most readily in culture, whilst Cell Line 1 proliferated least quickly. **(B)** Log transformed data, the analysis of which showed significant differences between the slopes (p = 0.005658). The sixth cell line was not cultured past 20 days for experimental reasons, therefore is not included.

3.3.2.3 Fluorescent labelling

Cultured bovine cells were transduced readily with both GFP and mCherry lentiviruses (Fig. 3.5). Transduction rates with GFP lentivirus improved with increasing ratios of GFP lentiviral particles to cells, with a transduction rate of 82% achieved when a ratio of 20 lentivirus particles to 1 bovine cell was used (Fig. 3.6). Following purification by FACS, GFP-expressing cells re-established in culture and after 2 weeks of expansion almost all cells continued to express GFP (Fig. 3.7). The long-term stability of the transduction was tested after 14 weeks of expansion and 58% of cells were noted to remain GFP-positive (Fig. 3.7(c)).

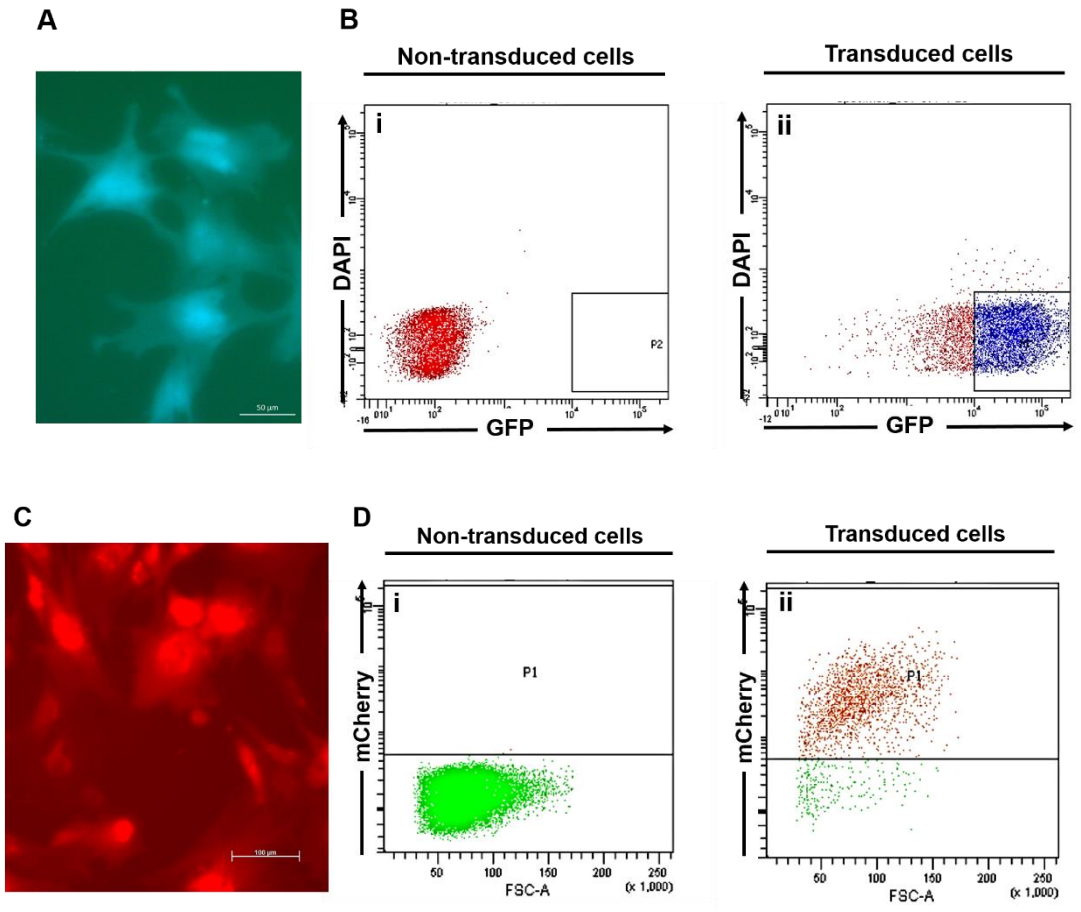


Figure 3.5. Lentiviral transduction of cultured bovine cells. (A) GFP-transduced bovine cells. Scale bar = 50µm. **(B)** FACS was used to collect a purified population of GFP-positive cells. **(i)** A non-transduced cell sample was used to gate for GFP-positive cells. **(ii)** The most highly fluorescent GFP-positive cells (blue dots) are in the small box. A ratio of 20:1 lentiviral particles to cells yielded a transduction rate of 82%. **(C)** Bovine cells expressing mCherry. Scale bar = 100µm. **(D)** FACS was also used to collect a purified population of mCherry-positive cells. No DAPI was used in this experiment. **(i)** Again, a non-transduced cell sample was used to gate for mCherry-positive cells. **(ii)** Cells positive for mCherry (orange dots) are in the top portion of the chart. A ratio of 5:1 lentiviral particles to cells yielded a transduction rate of 88.8%.

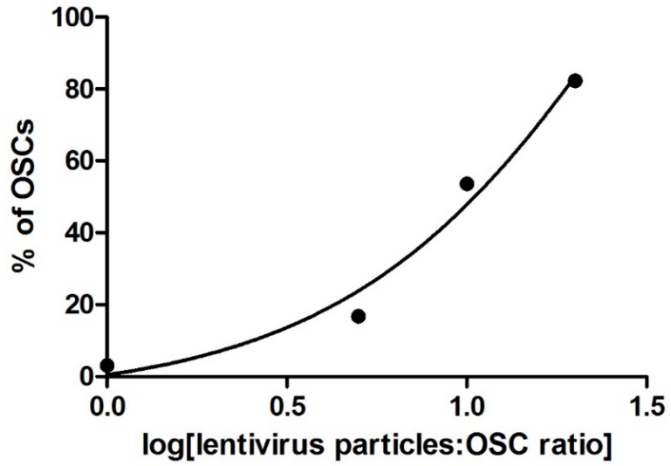


Figure 3.6. GFP-lentiviral transduction rates of cultured bovine cells. Transduction efficiency improved with increasing ratios of lentiviral particles to cells.

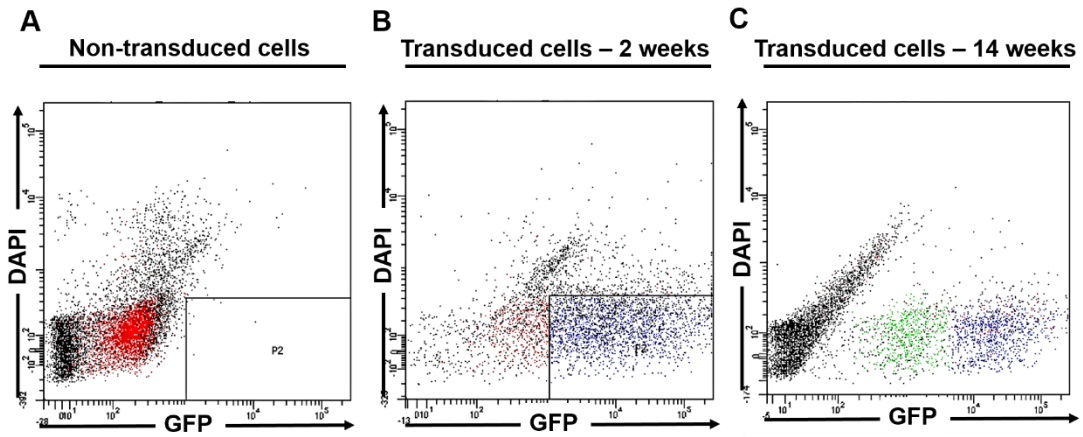


Figure 3.7. Lentiviral transduction demonstrated long-term stability in cultured bovine cells as shown by flow cytometry. (A) Non-transduced cells were analysed to gate for GFP-positive cells. **(B)** The vast majority of cells remained GFP-positive (blue dots) 2 weeks after transduction. **(C)** 58% of cells remained GFP-positive (blue dots) 14 weeks after transduction.

Transduction with mCherry lentivirus was more efficient, with a peak transduction rate of 88.8% utilising a 5:1 ratio of lentivirus particles to cells. Increasing the ratio

further did not improve transduction rates. Qualitative examination of expanded purified GFP- or mCherry-expressing cells revealed that lentiviral transduction appeared to negatively affect the mitotic activity and health of the cells, although this was not confirmed quantitatively when growth rates were statistically analysed (Fig. 3.8). Furthermore, the FACS process for purification appeared detrimental to the health of the cells, as high levels of cell death were subjectively seen immediately post-purification.

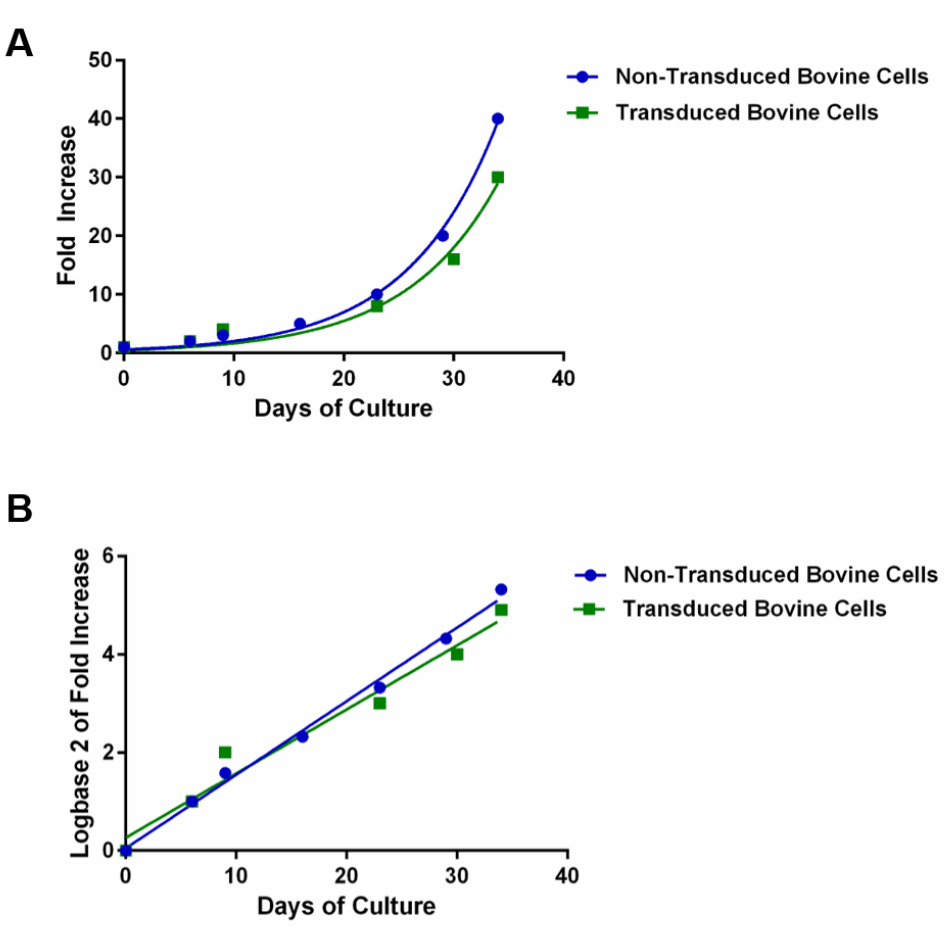


Figure 3.8. GFP lentiviral transduction did not significantly negatively affect the growth rate of cultured bovine cells compared to non-transduced cells. (A) Non-logarithmically transformed data illustrated that non-transduced cells appeared to grow faster than transduced cells; although statistical analysis of transformed data **(B)** demonstrated no significant difference between the slopes ($p = 0.1532$).

As a result, labelling with rhodamine-conjugated dextrans was attempted as an alternative method of fluorescent labelling (Fig. 3.9). This approach was successful, with no effect on cell growth rates when compared with non-dextrans exposed cells. Cells were not purified for further use due to the deleterious effect of FACS on cell health observed previously.

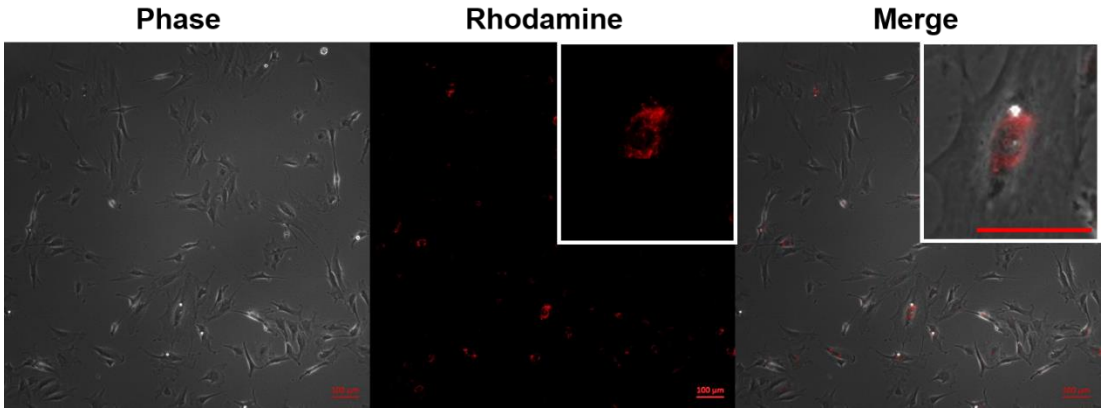


Figure 3.9. Cultured bovine cells could be fluorescently labelled with rhodamine-conjugated dextrans. An enlarged image of a dextrans-labelled cell is shown in the white boxes, demonstrating a perinuclear punctate expression. Scale bar = 100 µm.

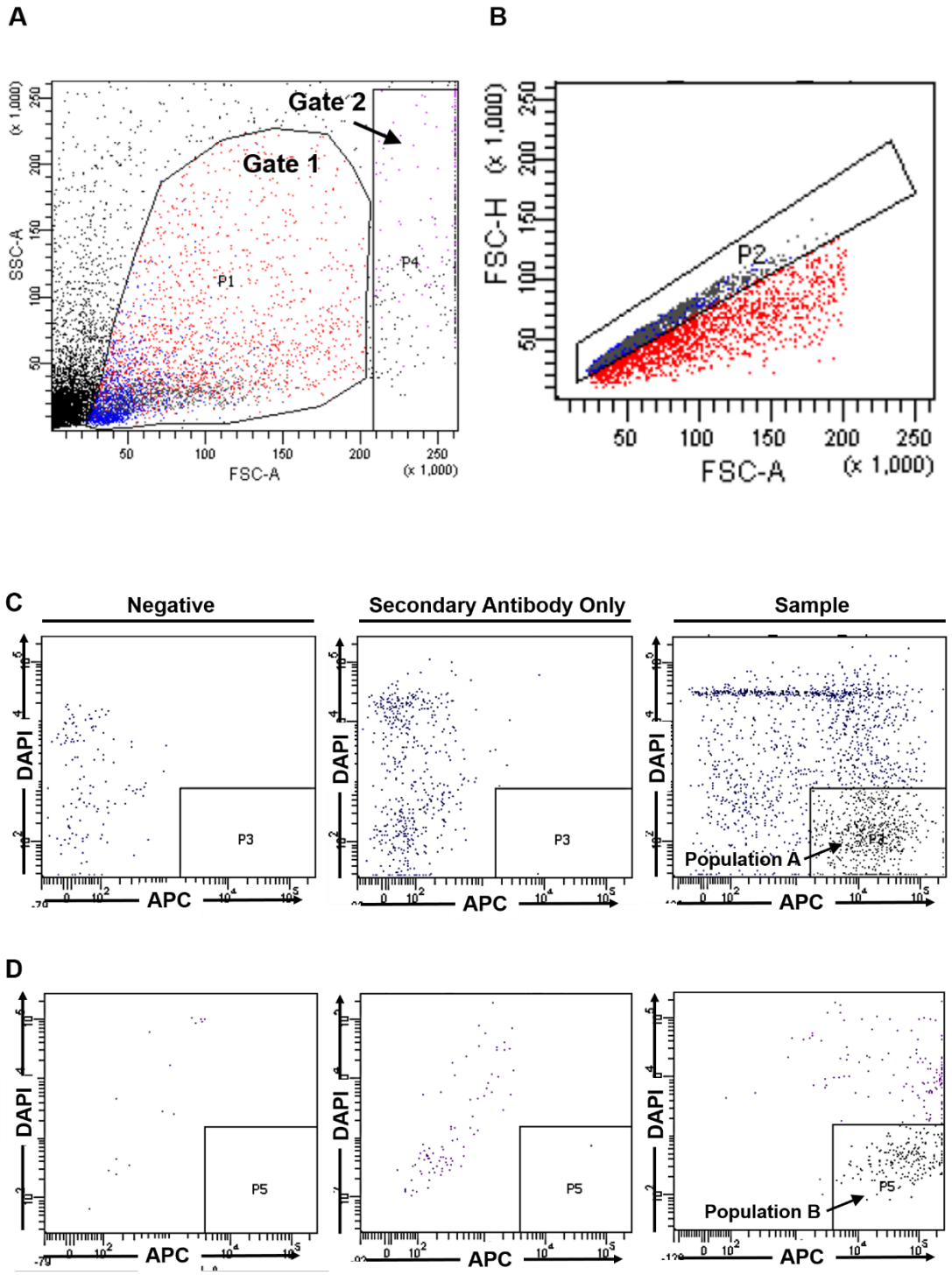
3.3.3 Isolation and culture of human DDX4-positive cells

3.3.3.1 Cell isolation

After optimisation of the cell sorting protocol using bovine tissue, experiments were commenced on human ovarian cortex. Two to four pieces of human tissue were used per patient, per experiment. All tissue was cryopreserved at the time of biopsy and subsequently thawed when required. As detailed in Chapter 2, the patients comprised one teenager undergoing ovarian cortex cryopreservation prior to chemotherapy, five women undergoing elective Caesarean section and two women undergoing ovarian cortex cryopreservation having previously had non POI-inducing chemotherapy. The use of 600 u/ml of collagenase caused over-dissociation of human tissue, with substantial cell death as detected by the use of DAPI during flow cytometry; therefore

we reverted to using 400 u/ml. The DNase I: collagenase ratio of 1:100 was maintained. As with the bovine experiments, once the protocol was modified, DDX4-positive cells were consistently isolated from adult human ovarian cortex (n = 8; Fig. 3.10). Two discrete populations, Populations A and B, were detected as in the bovine experiments, and constituted only a small number of the mixed cell population, with an average yield of 1.9 ± 0.6% (± S.E.M.) and 1.4 ± 0.7% (± S.E.M.) for Populations A and B, respectively.

Figure 3.10. Representative FACS plots for dissociated human ovarian cortex. **(A)** Intact cells were gated from all events. Gate 1 contained smaller cells than Gate 2. **(B)** Single cells were subsequently gated for and used for fluorescent analyses. **(C)** Population A cells were derived from Gate 1. No APC-positive cells (and therefore DDX4-positive cells) were detected if the cell suspension has no antibodies (negative) or the secondary antibody only. DAPI staining allowed for exclusion of dead cells such that live, DDX4-positive cells were found in the bottom right hand box. **(D)** Population B cells are derived from Gate 2. Again, no APC-positive cells were detected in the two negative controls and live, DDX4-positive cells were located in the bottom right hand box.


A
B
Gate 2
Gate 1
P1
P4
SSC-A
FSC-A
(x 1,000)
FSC-H
P2
C
Negative
Secondary Antibody Only
Sample
DAPI
APC
P3
Population A
D
P5
Population B

In terms of raw numbers of cells, this equated to a range of 21 - 2296 for Population A and 0 - 2024 for Population B (Table 3.3).

Table 3.3. Raw numbers of DDX4-positive cells isolated during each human experiment with primary outcome of establishment in culture.

Patient	Age (years)	Population A (no. of cells)	Established in culture	Population B (no. of cells)	Established in culture
1	13	78	N	102	Y
2	22	165	N	274	Y
3	32	365	N	67	Y
4	33	276	Y	91	Y
5	33	21	N	2	N
6	38	10	N	14	N
7	39	162	N	0	N
8	40	2296	Y	2024	Y

There was no apparent correlation between the number of cells isolated and the age of the patient, although it is a small sample size (Fig. 3.11). There did appear to be an association between the source of the tissue and cell yield, however, with samples from women undergoing ovarian cortex cryopreservation yielding more cells than from the pregnant patients, with the notable exception of Patient 8. Indeed, the highest cell yields were obtained from Patient 8 who was noted to have more friable tissue on thawing and thus the cortex was more easily dissociated.

Due to tissue availability, the new dissociation protocol was not performed on human tissue and, due to low cell yields, all freshly isolated cells were collected for *in vitro* culture instead of characterisation.

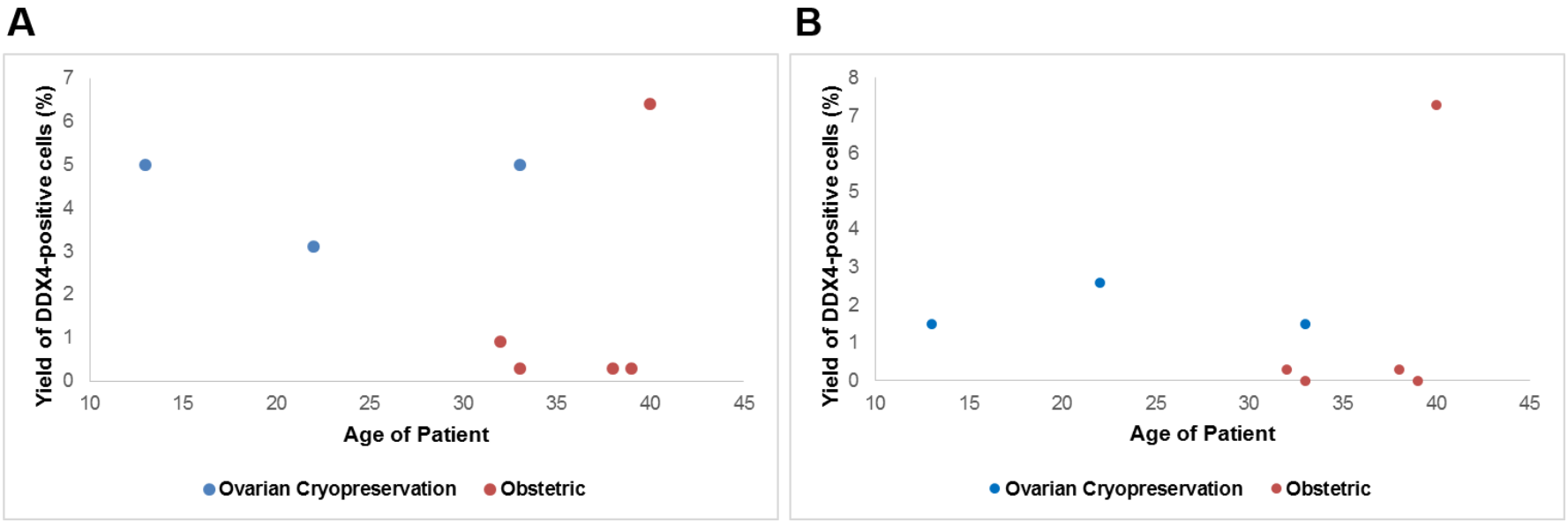


Figure 3.11. Scatterplots of the yield of DDX4-positive cells in each patient according to their age and pathology. (A) Population A yields were higher in the patients undergoing ovarian cortex cryopreservation (blue dots), with the exception of the 40 year old obstetric patient (Patient 8). **(B)** The same pattern applied to Population B cells.

3.3.3.2 *In vitro* culture of isolated cells

Population B cells from 5 of the patients became established in culture after 3 - 14 days *in vitro* (Table 3.3). They reached confluence and were ready for passage 11 - 28 days later. The tissue of the other 3 patients appeared to be over-dissociated, with high levels of cell death seen during FACS. This resulted in lower absolute numbers of cells being collected, thus possibly explaining why these cells did not establish in culture. Population A cells established on two occasions, from Patients 4 and 8. The time they took to establish and the time to first passage was comparable to Population B. No feeder layer was required for the establishment and growth of either population. As seen in the bovine cells, the human cells initially grew in colony-like structures, but grew more evenly following the first passage. The human cells also had the same elongated morphology as the bovine cells (Fig. 3.12(a)) and, as seen in the bovine cultures, the two different human populations looked identical microscopically. Isolated cells from Patient 4 have been cultured continuously *in vitro* for 7 months, undergoing more than 20 passages. Live cell imaging of Patient 2-derived cells demonstrated amoeboid-like movement of the cells across the base of the culture well (see video submitted with thesis). Floating spherical cells of 30-40 µm in diameter were also seen to be spontaneously generated from human cells (Fig. 3.12(b)). Again, this observation was rare, with the incidence estimated to be similar to that of the bovine OLCs (i.e. at least one OLC and no more than ten OLCs during the entire *in vitro* culture period of each cell line and only in the later passages).

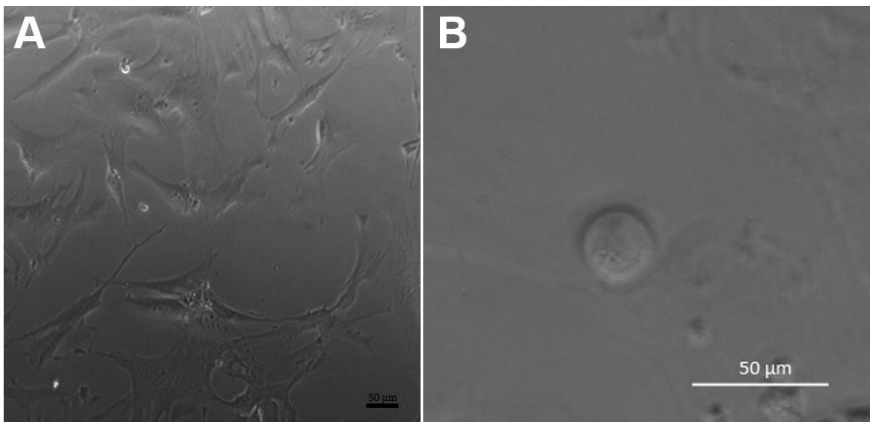


Figure 3.12. Human cells cultured *in vitro*. (A) Cultured human cells were elongated in shape and maintained their morphology during long-term culture (Patient 2, passage 10). **(B)** An example of an infrequently observed OLC spontaneously formed during *in vitro* culture. Scale bars = 50µm.

Statistically significant variable rates of growth have been observed between patients (p = 0.001738; Fig. 3.13). This is not accounted for by age or pathology. Of note, although Population A cells derived from Patient 8 had identical growth to Population B cells, Patient 4-derived Population A cells grew more slowly than their Population B counterparts and ceased to proliferate after passage 14. Furthermore, human-derived cells appeared to have a slower rate of proliferation than bovine-derived cells (p = 0.0043; Fig. 3.14).

Human cells could also be cryopreserved, with successful thawing possible when DMSO was used as a cryoprotectant.

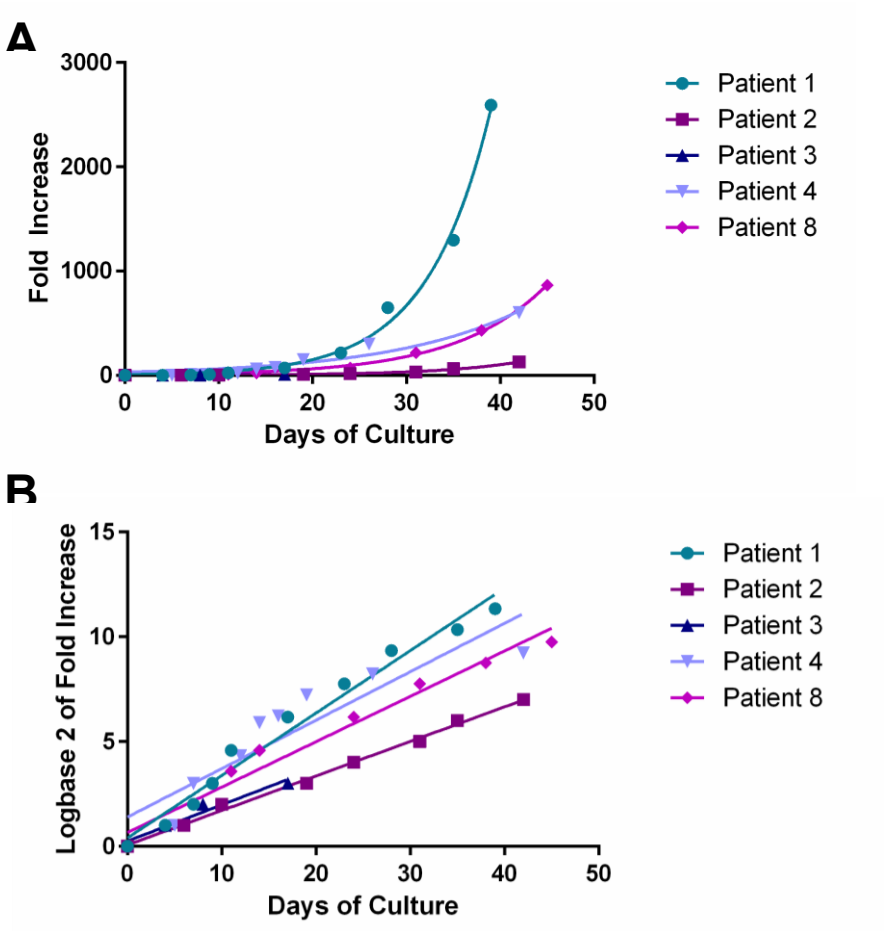


Figure 3.13. Growth rates of cultured human cells. (A) Human cell lines exhibited variable rates of growth during *in vitro* culture. **(B)** This was confirmed statistically by analysis of transformed data, with the slopes being significantly different from each other (p = 0.001738).

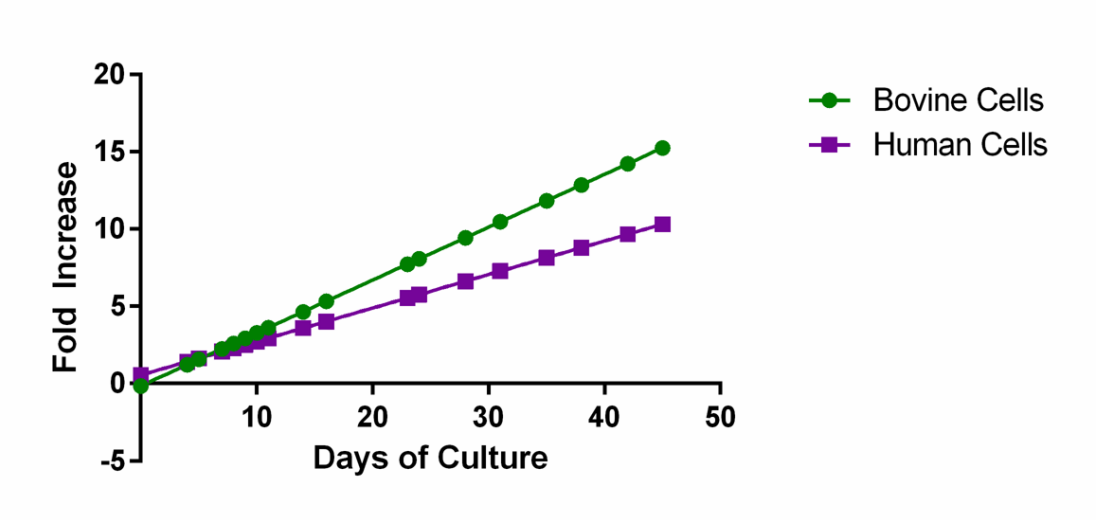


Figure 3.14. Comparison of the mean growth rates of bovine and human cells revealed that bovine cells had a significantly higher growth rate than human cells (*p* = 0.0043).

3.3.3.3 Fluorescent labelling

Cultured human cells were less readily transducible with fluorescently-labelled lentiviruses than bovine cells, with FACS determining a transduction rate of 36% using a 20:1 GFP lentivirus particles to cultured cell ratio (compared with 82% in bovine cells) and 60.8% using a 5:1 mCherry lentivirus particles to cell ratio (compared with 88.8% in bovine cells). An increased mCherry ratio of 10:1 resulted in high cell death rates as determined by DAPI staining during FACS. Both GFP and mCherry expression were clearly visible in transduced cells under an inverted microscope (Fig. 3.15). As with the transduced bovine cells, purified transduced human cells propagated much more slowly than non-transduced cells ($p = 0.04245$; Fig. 3.16).

Rhodamine-conjugated dextrans labelling was an effective substitute with no apparent effect on proliferation rates when compared with non-dextrans exposed cells.

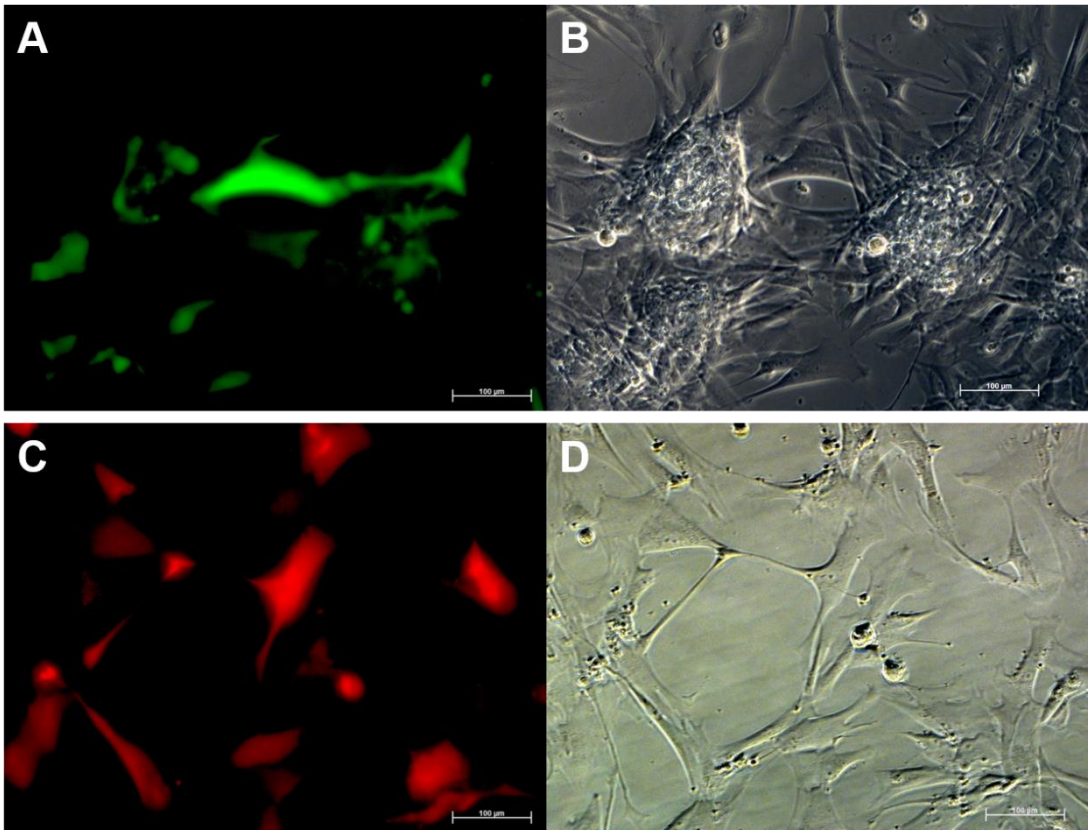


Figure 3.15. Lentiviral transduction of cultured human cells. Cells could be transduced to express GFP **(A)** and mCherry **(C)**. **(B)** and **(D)** Corresponding phase images demonstrating limited transduction efficiency.

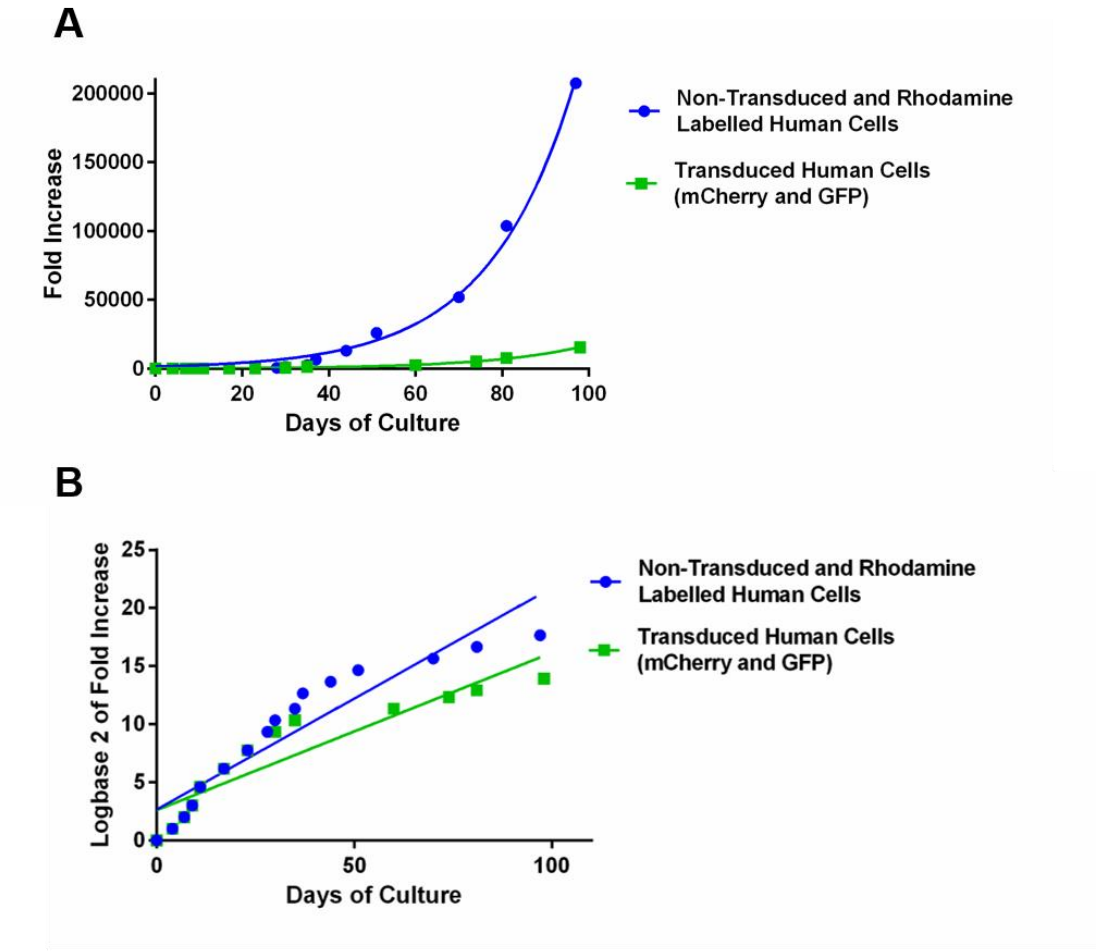


Figure 3.16. Lentiviral transduction with either GFP or mCherry was detrimental to the growth rate of cultured human cells compared to non-transduced and rhodamine-labelled cells (A). (B) Statistical analysis of transformed data showed that the slopes were significantly different ($p = 0.04245$).

3.4 Discussion

3.4.1 Isolation of cell populations using DDX4-based FACS protocol

The data detailed in this chapter demonstrate that, once the published protocol (White *et al.*, 2012, Woods and Tilly, 2013) is optimised for use with the tissue of interest, a scarce number of cells can be isolated from bovine and human ovarian cortex using a DDX4-based FACS technique. The finding of such cells has not been published in the cow previously and, at the time of the experiments, had only been reported by one group in humans (White *et al.*, 2012). The cells detected as being DDX4-positive comprised a rare population within the dissociated cortex, in keeping with previous studies (Pacchiarotti *et al.*, 2010, White *et al.*, 2012, Hernandez *et al.*, 2015). The prevalence of small DDX4-positive cells detected by immunohistochemistry was similarly low, although it is unclear whether these are the same cells as were detected by FACS.

In the human experiments, there did not seem to be a correlation between the age of the patient and cell yield, despite previous observations of a decline in prevalence with age in mice (Pacchiarotti *et al.*, 2010). With regards pathology of the donating patient, all women undergoing elective Caesarean section, bar one, had lower yields than those patients who were undergoing ovarian cortex cryopreservation. It may be that the hormonal milieu of pregnancy suppresses the proliferation of these cells; alternatively, it could be that the illnesses or treatment endured by the cryopreservation patients have stimulated their proliferation. With regards the latter, it has recently been shown that women, such as Patient 2, who have been exposed to the ABVD regimen of chemotherapy (comprising adriamycin, bleomycin, vinblastine and dacarbazine) have significantly increased numbers of non-growing follicles (NGFs) when compared with age-related predicted values (McLaughlin *et al.*, 2015, McLaughlin *et al.*, 2016). Furthermore, follicle clusters similar to those seen in fetal and pre-pubertal ovaries are observed in these patients. The underlying reason for this finding has not been elucidated, but one possible explanation is that the treatment has stimulated OSC proliferation and differentiation into new follicles. With respect to the patients undergoing elective Caesarean section, Patient 8 was the clear exception: it may be that the dissociation of her tissue was much more effective due to its friable nature, for which the cause is unknown. As the bovine experiments involved pooling of tissue

from several heifers of unknown age and health, a similar analysis was not possible for the cow. Of note, it is clinically valuable that the cells can be isolated from cryopreserved ovarian cortex as it means that patients' tissue could be stored until the cells were required.

There was only one observable difference between the isolation experiments in the two species: bovine ovarian cortex required a higher concentration of enzymes for adequate disaggregation, possibly due to the stroma being more dense than human ovarian cortex. All other aspects of the method yielded similar results, with two populations of DDX4-positive cells detected in both bovine and human tissue. The identification of two populations has been reported in pigs (Bui *et al.*, 2014), although a purified population was not examined in this study. No studies analysing purified cell populations have reported more than one population to date. The differences between these two populations, other than in cell size and prevalence, is yet to be ascertained. It is possible Population B, which is larger in cell size, is at a different stage of the cell cycle than Population A: proliferating cells are largest at the end of interphase just prior to mitosis, when they have doubled the amount of their DNA in preparation for division (Cooper, 2000). Unfortunately, poor cell yields meant that ploidy analysis of the isolated cells was not possible. Another possible explanation is that Population B is a less pure population than Population A: if the cells are in aggregates, then they will appear larger in size during flow cytometry. This may also explain why Population B established more often in culture than Population A, as the Population B may have contained "helper" cells that encouraged initiation of growth *in vitro*. One hypothesis would be that these "helper" cells may be located next to putative OSCs within the ovarian cortex, possibly as part of a niche, and their presence contributes to the regulation of the normal function of the putative OSCs.

With regards to cell size, the bovine cells were, on average, approximately 14-16 µm in diameter. Although the size of the human cells was not calculated due to tissue availability, the FSC values for the two species were very similar, indicating that they are of similar size. This finding is in keeping with that of previous groups: the mouse cells isolated by Wu's group by MACS for either Ddx4 or Ifitm3 were 12-20 µm in diameter (Zou *et al.*, 2009, Zou *et al.*, 2011), whilst the putative OSCs reported by Pacchiarotti *et al*. were 10-15 µm in diameter (Pacchiarotti *et al.*, 2010). However, the cells are larger than those reported by White *et al*., who found that the human cells

they isolated were 5-8 µm (White *et al.*, 2012). It is difficult to ascertain whether these differences could be a result of different methods of measuring the cells, or whether different populations of cells are being isolated.

An attempt was made to investigate whether IFITM3 could be used to isolate a similar population of bovine cells, as it had been validated for use by one previous group at the time of the experiment (Zou *et al.*, 2011). This was not successful and due to time limitations it was not repeated; however, optimisation of this technique may prove productive and would be a valid future study.

Finally, in relation to the isolation data in this chapter, it is evident that the methodology hinges on adequate dissociation of the tissue and correct FACS gating strategies. There is a balance between disaggregating the tissue and causing cell death by exposure to dissociative enzymes. An altered disaggregation protocol greatly increased the raw cell numbers obtained by decreasing the exposure to the enzymes but still allowing adequate disaggregation. The prevalence of the cells also appeared to increase, however, which indicates either that the initial methodology wasn't detecting all the DDX4-positive cells, or that the new protocol is selecting a less pure population. Regarding the gating strategy, Population B was initially not detected but it was identified once the gating was adjusted. It is therefore highly possible that the inability to recapitulate the sorting experiments (Zhang *et al.*, 2015) could be a result of the gating strategy used.

3.4.2 *In vitro* culture of isolated cells

Isolated cells established in culture within 2 weeks and were ready for passage 1 week (bovine) or up to 4 weeks (human) later. This is a shorter timeframe than that reported previously, where proliferating putative human OSCs were not detected until 4 - 8 weeks after FACS (White *et al.*, 2012). This was also despite the fact that a feeder layer was not used, which had previously been reported as being required for the cells to initiate growth (Zou *et al.*, 2009, Pacchiarotti *et al.*, 2010, Hu *et al.*, 2012, White *et al.*, 2012, Bui *et al.*, 2014, Zhou *et al.*, 2014, Hernandez *et al.*, 2015, Xiong *et al.*, 2015, Lu *et al.*, 2016). This indicates that the criticism that *in vitro* transformation is occurring in these cells (Oatley and Hunt, 2012) may be less likely. In addition, the lack of a feeder layer (which is normally derived from mouse), means that there is no interspecies contamination which would be required if such cells were to be used

clinically. One group has successfully used human umbilical cord mesenchymal stem cells as a feeder layer (Lu *et al.*, 2016), but this would still result in the use of tissue from another human.

The cell clusters, with blurred boundaries, that initially formed after isolation during these experiments were similar to those previously described (Zou *et al.*, 2009), as was the morphology of the cells (Hu *et al.*, 2012, Hernandez *et al.*, 2015), being elongated and spindle-like. However, this is in contrast to the morphology reported by others where the cells have been described as round with a large nuclear-to-cytoplasm ratio (Zou *et al.*, 2009, Pacchiarotti *et al.*, 2010, Bui *et al.*, 2014, Zhou *et al.*, 2014, Xiong *et al.*, 2015, Lu *et al.*, 2016). However, it is often unclear from the literature whether the morphology that is described is the shape of the cell immediately after isolation, or whether it is what is observed in culture. Furthermore, the morphology of these cells could perhaps be affected by the presence of a feeder layer: if feeder layers encourage colony growth rather than more even growth across the plate, then perhaps the morphology of the isolated cells is not as apparent.

Bovine and human cells could be cultured *in vitro* long-term although there were both intra- and interspecies variations in growth rates. Different patients exhibited different rates of growth, and although the youngest patient appeared to have the most rapidly proliferating cells, there did not seem to be any correlation between growth rate and either age or pathology of the patient. The different bovine cell lines also demonstrated variable rates of growth, although the rates were not as significantly different as seen in the human cells: the bovine tissue was pooled, thus possibly masking any effect age or inter-individual variability may have had. Bovine cells had higher growth rates than human cells. It may be that it is an interspecies variation, however, as the culture medium for both species contained FBS, it is possible that this provided a more conducive environment for the proliferation of bovine cells. It may be that substituting FBS for human serum albumin, human serum substitute or human adult serum (if available) would improve human cell growth rates. Cells from both species could be successfully cryopreserved which is important for long-term maintenance of a cell line for ongoing experimentation. Moreover, if these cells are be used clinically, it would be mean that cells could be stored for protracted periods of time, until the patient requires them.

As seen in previous studies, both bovine and human cultured cells spontaneously generated larger cells with an oocyte-like morphology (Pacchiarotti *et al.*, 2010, White *et al.*, 2012, Hu *et al.*, 2012, Bui *et al.*, 2014, Zhou *et al.*, 2014, Hernandez *et al.*, 2015). These OLCs were observed very infrequently. The molecular nature of these cells will be explored further in Chapter 4.

3.4.3 Fluorescent labelling of cultured isolated cells

In contrast to previous OSC research (Zou *et al.*, 2009, White *et al.*, 2012), cells were fluorescently labelled using a lentivirus rather than a retrovirus. Lentiviruses are a retrovirus subtype that have the advantage over retroviruses of being able to transduce non-dividing cells (Naldini *et al.*, 1996). In this research, lentivirus transduction was chosen as fluorescently labelled viruses that had been validated for use in stem cells were locally available. Cultured cells could be labelled successfully by lentiviral transduction, with bovine cells being more readily transduced than human. This may be because bovine cells were more susceptible to lentiviral cell membrane fusion, or perhaps because the lentivirus was more effective at transducing more rapidly proliferating cells. In order for fluorescent cells to be used in future functional assessments, a pure population was preferable. Unfortunately, FACS-based purification of the transduced cells appeared to be detrimental to cell health and therefore the observed long-term stability of the transduction was desirable as it avoided the need for repeated cell sorting for purification. However, qualitatively (and, in the case of the human cells, quantitatively) it became evident that GFP-transduced cells were not proliferating as quickly as non-transduced cells. It was hypothesised that as the form of GFP used in the lentivirus (humanised recombinant GFP or hrGFP) can form dimers (Day and Davidson, 2009), it may be too metabolically demanding for the cells to translate the protein and thus they have less energy available for mitosis. A mCherry-containing lentivirus was then evaluated as, being a monomer (Day and Davidson, 2009), it was thought that it might be less energy-depleting for the cells. Unfortunately, mCherry transduction was equally damaging to human cell growth rates.

Due to concerns about the health of lentiviral-transduced cells, an alternative method of labelling the cells was attempted which would not require the cell to perform any protein translation. Fluorescently-tagged dextrans are inert hydrophilic

polysaccharides which are endocytosed by the cell and are contained within intracytoplasmic vesicles. Their fluorescence does not rely on the cell to perform any biological processes and their use in tracing cells is well-established (Gimlich and Braun, 1985). Labelling of the cultured cells from both species with rhodamine dextrans was straightforward and did not appear to have a detrimental effect on proliferation rates. This appears to be a useful and novel approach to fluorescently labelling such cells, with no other reports of dextran use in putative OSCs in the literature.

3.4.4 Summary

In summary, two rare populations of cells were isolated from adult bovine and human ovarian cortex using a DDX4-based protocol, with one being smaller in size, but greater in prevalence than the other. The cells established more rapidly in culture than previously reported, but have similar morphological features to some studies. Bovine cells proliferated more quickly than human cells and there appeared to be variation in human cell growth that was not related to age or underlying pathology. The cells could be fluorescently labelled in a stable manner, with fluorescent dextrans having no apparent effect on cell proliferation. In order to determine whether these cells were putative OSCs are not, it was important to characterise them and evaluate their molecular signature, which is presented in the next Chapter.

Chapter 4

Characterisation of Putative OSCs

4.1 Introduction

4.1.1 The Molecular Characteristics of an OSC

As yet, no OSC-specific marker has been ascertained; instead, groups that have isolated putative OSCs have used a series of different markers to provide evidence of the cell's dual germline and pluripotent nature. Of note, a robust specific marker has not yet been elucidated even in the more widely studied SSC (Hermann *et al.*, 2011) and investigators in this field have used a similar approach of utilising a combination of germline and pluripotent markers to characterise this population of stem cells (Kolasa *et al.*, 2011). Similar to OSCs, the scarcity of SSCs in adult testicular tissue has limited research, especially in humans, with the first stable human SSC line only derived very recently (Hou *et al.*, 2015). With regards OSCs, research has demonstrated that the cells both transcribe and translate germline and stem cell markers, with both mRNA and protein expression being reported.

4.1.1.1 mRNA expression

A range of different genes have been analysed by the groups who have isolated pure populations of putative OSCs to date, with some groups publishing more comprehensive characterisation profiles than others (Table 4.1). The most commonly used pluripotency markers have been *POU5F1* and *NANOG* and the most frequently used germline markers have been *DDX4*, *PRDM1*, *DPPA3* and *IFITM3* (Zou *et al.*, 2009, Pacchiarotti *et al.*, 2010, Zou *et al.*, 2011, Hu *et al.*, 2012, White *et al.*, 2012, Wolff *et al.*, 2013, Wolff *et al.*, 2014, Bui *et al.*, 2014, Zhou *et al.*, 2014, Hernandez *et al.*, 2015, Xiong *et al.*, 2015, Lu *et al.*, 2016). The analysis of both freshly isolated and cultured cells is important: this allows both an assessment of whether the DDX4 antibody is truly selecting DDX4-positive cells and some investigation of whether *in vitro* transformation in culture is occurring. However, only three groups have reported the analysis of mRNA expression of freshly isolated putative OSCs (Pacchiarotti *et al.*, 2010, White *et al.*, 2012, Hernandez *et al.*, 2015) and only two of these groups have examined both freshly isolated and cultured OSCs, with results indicating that a change in molecular signature does not occur in culture (White *et al.*, 2012, Hernandez *et al.*, 2015).

Table 4.1. Table comparing the mRNA expression of putative OSCs reported in the literature to date. Grey boxes indicate that the marker was not analysed by the named group.

Marker (mRNA)	Group									
	Zou *et al.* (2009 and 2011)	Pacchiarotti *et al.* (2010)	Hu *et al.* (2012)	White *et al.* (2012)	Wolff *et al.* (2013 and 2014)	Zhou *et al.* (2014)	Bui *et al.* (2014)	Hernandez *et al.* (2015)	Xiong *et al.* (2015)	Lu *et al.* (2016)
POU5F1	✓	✓	✓			✓	✓			
LIN28										
NANOG	X		✓		✓	X	✓			
PRDM1	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
DPPA3	✓			✓	✓			✓		
IFITM3	✓			✓	✓	✓	✓	✓	✓	✓
C-KIT	X	✓				X	✓			
DDX4	✓	✓		✓		✓	✓	X	✓	✓

Overall, there are many similarities between the gene expression of the putative OSCs as assessed by different groups, with consistent expression of *Pou5f1*/*POU5F1* (Zou *et al.*, 2009, Pacchiarotti *et al.*, 2010, Zou *et al.*, 2011, Hu *et al.*, 2012, Bui *et al.*, 2014, Zhou *et al.*, 2014), *Prdm1*/*PRDM1* (Zou *et al.*, 2009, Pacchiarotti *et al.*, 2010, Hu *et al.*, 2012, White *et al.*, 2012, Wolff *et al.*, 2013, Bui *et al.*, 2014, Wolff *et al.*, 2014, Zhou *et al.*, 2014, Xiong *et al.*, 2015, Lu *et al.*, 2016), *Dppa3*/*DPPA3* (Zou *et al.*, 2009, White *et al.*, 2012, Wolff *et al.*, 2013, Wolff *et al.*, 2014, Hernandez *et al.*, 2015) and *Ifitm3*/*IFITM3* (Zou *et al.*, 2009, Zou *et al.*, 2011, White *et al.*, 2012, Wolff *et al.*, 2013, Bui *et al.*, 2014, Wolff *et al.*, 2014, Zhou *et al.*, 2014, Hernandez *et al.*, 2015, Xiong *et al.*, 2015, Lu *et al.*, 2016). The expression of *Nanog*/*NANOG* and *Ddx4*/*DDX4* has shown variability, however. Wu's group were unable to detect *Nanog* in cultured OSCs of either the mouse (Zou *et al.*, 2009, Zou *et al.*, 2011) or rat (Zhou *et al.*, 2014), which is in contrast to findings by other groups (Hu *et al.*, 2012, Wolff *et al.*, 2013, Wolff *et al.*, 2014, Bui *et al.*, 2014). Furthermore, as discussed in section 1.2.5.2, one group did not detect *Ddx4/DDX4* in either freshly isolated or cultured mouse or human cells, despite the DDX4 protein being utilised for cell isolation (Hernandez *et al.*, 2015).

4.1.1.2 Protein expression

Almost all reports of putative OSC isolation have demonstrated that the detected mRNA can be translated into protein (Zou *et al.*, 2009, Pacchiarotti *et al.*, 2010, Zou *et al.*, 2011, Hu *et al.*, 2012, White *et al.*, 2012, Bui *et al.*, 2014, Zhou *et al.*, 2014, Xiong *et al.*, 2015, Lu *et al.*, 2016). Again, some groups have performed more thorough characterisation than others, with the expression of between one (Zhou *et al.*, 2014) and six (Zou *et al.*, 2009, Bui *et al*., 2014) proteins being examined (Table 4.2).

Table 4.2. Table comparing the protein expression of putative OSCs reported in the literature to date. Grey boxes indicate that the marker was not analysed by the named group.

Marker (Protein)	Group									
	Zou *et al.* (2009 and 2011)	Pacchiarotti *et al.* (2010)	Hu *et al.* (2012)	White *et al.* (2012)	Wolff *et al.* (2013 and 2014)	Zhou *et al.* (2014)	Bui *et al.* (2014)	Hernandez *et al.* (2015)	Xiong *et al.* (2015)	Lu *et al.* (2016)
POU5F1	✓	✓	✓			✓	✓			
LIN28			✓							
NANOG	X	✓	✓							
PRDM1	✓			✓			✓			
DPPA3	✓			✓			✓			
IFITM3	✓			✓			✓		✓	✓
C-KIT		✓					✓			
DDX4	✓		✓				✓	X	✓	✓
DAZL										

There have been no reports asserting any differences between mRNA expression of OSC markers and protein expression: once again, Pou5f1/POU5F1 (Zou *et al.*, 2009, Pacchiarotti *et al.*, 2010, Hu *et al.*, 2012, Bui *et al.*, 2014, Zhou *et al.*, 2014), Prdm1/PRDM1 (Zou *et al.*, 2009, White *et al.*, 2012), Dppa3/DPPA3 (Zou *et al.*, 2009, White *et al.*, 2012, Bui *et al.*, 2014) and Ifitm3/IFITM3 (Zou *et al.*, 2009, Zou *et al.*, 2011, White *et al.*, 2012, Bui *et al.*, 2014, Xiong *et al.*, 2015, Lu *et al.*, 2016) are consistently detected, whereas Nanog/NANOG and Ddx4 expression is more variable. As seen with the mRNA data, Wu's group could not detect Nanog protein in mouse OSCs (Zou *et al.*, 2009), which is in disagreement with other groups (Pacchiarotti *et al.*, 2010, Hu *et al.*, 2012). With regards to Ddx4, Hernandez *et al*. were also unable to demonstrate its expression at the protein level in the mouse, although no data for human OSCs were described (Hernandez *et al.*, 2015).

In terms of detection methodology, all groups bar one have demonstrated protein expression by immunocytochemical methods. To date, only Hernandez *et al*. have utilised Western blotting to analyse protein expression in putative OSCs; however, its use was restricted to cultured mouse cells and only the presence of Ddx4 was examined (Hernandez *et al.*, 2015).

4.1.2 Evidence for spontaneous *in vitro neo*-oogenesis

A few groups have reported the spontaneous formation of OLCs *in vitro* (Pacchiarotti *et al.*, 2010, White *et al.*, 2012, Bui *et al.*, 2014, Hernandez *et al.*, 2015) as characterised by both morphology and oocyte-specific markers (Pacchiarotti *et al.*, 2010, White *et al.*, 2012) and morphology alone (Bui *et al.*, 2014, Hernandez *et al.*, 2015). Pacchiarotti *et al*. and White *et al*. both demonstrated mRNA expression of several oocyte-specific genes including *Gdf-9*/*GDF-9* (Pacchiarotti *et al.*, 2010, White *et al.*, 2012) and *Nobox*/*NOBOX* (White *et al.*, 2012) at the mRNA levels and White *et al*. confirmed the expression of other oocyte markers (YBX2 and LHX8) at the protein level using immunofluorescence (White *et al.*, 2012).

Some groups have also examined the presence of oocyte-specific markers in putative OSCs, with varied results. White *et al*. reported that markers such as *Gdf-9*/*GFP-9*,

Nobox/*NOBOX* and *Zp3*/*ZP3* could not be detected in freshly isolated cells but could be demonstrated in cultured mouse and human cells, indicating that the isolated DDX4-positive cells were not oocytes and that *in vitro neo*-oogenesis in culture must be occurring (White *et al.*, 2012). Yet, other studies have failed to detect markers of oocytes in cultured rodent OSCs (Zou *et al.*, 2009, Zhou *et al.*, 2014, Xiong *et al.*, 2015, Lu *et al.*, 2016).

4.1.3 Aims of this Chapter

The primary aim of the experiments described in this Chapter was to identify the expression pattern of the cells isolated from bovine and human ovarian cortex, in order to assess if they showed the pattern expected of putative OSCs. Prospective molecular markers for OSCs (detailed in section 1.2.2) were chosen based on previous studies on OSCs and the literature. The objective was to investigate the expression of these markers in freshly isolated and cultured cells at both the mRNA and protein level by the use of RT-PCR, immunocytochemistry and Western blotting. Lentiviral-transduced cells were also analysed for protein expression of the fluorescent markers, GFP and mCherry, to confirm that the transduced cells were translating the integrated DNA. The secondary aim was to analyse the cells and OLCs for the expression of oocyte-specific markers, thus allowing it to be determined whether spontaneous *in vitro neo*-oogenesis was occurring or not.

4.2 Materials and Methods

4.2.1 RT-PCR

4.2.1.1 Cultured cells

Cultured bovine and human cells were trypsinised, centrifuged for 5 mins at 800 x *g* and the cell pellet resuspended in 350µl RLT buffer containing 1% (v/v) β-ME. Total RNA was extracted, cDNA was synthesised and RT-PCR was performed as per sections 2.9.1, 2.9.2 and 2.9.3. Tables 2.4 and 2.5 detail the primers used for characterisation of bovine cells and Table 2.6 details the primers used in human experiments. If possible, 500ng of RNA was used to synthesise cDNA. For earlier passages, where cell numbers were lower, it was not possible to obtain 500ng of RNA, therefore the whole sample of RNA was utilised.

4.2.1.2 Freshly isolated cells

The characterisation of freshly isolated cells was technically more complicated due to low cell yields. Two different disaggregation protocols, two different flow cytometry facilities and several different RNA extraction, cDNA synthesis and RT-PCR methods were tried in an attempt to generate reliable results (Table 4.3).

Table 4.3. Methodologies used to characterise freshly isolated cells. The initial disaggregation protocol (section 2.3.1) and modified protocol (2.3.1.1) were used at two different flow cytometry facilities (QMRI and SBS). The details and sources of the RNA extraction, cDNA synthesis and RT-PCR methods can be found in sections 2.9.1, 2.9.2, 2.9.3, 4.2.1.2.1 and 4.2.1.2.2). * This combination of methodologies yielded the cells used for characterisation as it provided the largest number of cells, collection directly into TRIzol was possible (this was not possible at QMRI due to restrictions at that facility) and RNA extraction was most efficient.

Species	Disaggregation Protocol	Flow Cytometry Facility	RNA Extraction Method	cDNA Synthesis Method	RT-PCR Method
Bovine	Initial	QMRI	RNeasy Micro Kit	Maxima	MyTaq™
Human	Initial	QMRI	ARCTURUS®	SuperScript® III	MyTaq™
Bovine	Initial	QMRI	ARCTURUS®	SuperScript® III	MyTaq™
Bovine	Modified	QMRI	TRIzol	Maxima	MyTaq™
Bovine *	Modified	SBS	TRIzol	Omniscript®	Taq DNA Polymerase

4.2.1.2.1 Initial disaggregation protocol

The initial disaggregation protocol (see section 2.3.1) yielded extremely low numbers of cells and, with the methods described in section 2.9.1, RNA concentrations were very low. Therefore, a different method of RNA extraction was used in an attempt to attain higher concentrations of RNA. Bovine and human cells (n = 1 for each species) were collected in 1ml of PBS containing 10% (w/v) BSA and 0.5M EDTA and RNA was extracted immediately using the ARCTURUS® PicoPure® RNA Isolation kit

(Applied Biosystems, Life Technologies) as per the manufacturer's instructions. On-column DNase I digestion was performed for 15 mins and total RNA was eluted in the kit's Elution Buffer. Total RNA concentrations were measured using a Nanodrop ND-1000 spectrophotometer as per section 2.9.1. RNA concentrations were low (< 13µg/µl), therefore all of the RNA was utilised for cDNA synthesis. First strand cDNA synthesis was performed using the Superscript® III First-Strand Synthesis System as per the manufacturer's instructions and a PTC-100 Thermal Cycler was used with the cycling conditions described in Table 4.4.

Table 4.4. First strand cDNA synthesis Thermocycler programme for SuperScript® System.

Step	Temperature (ºC)	Time (mins)
1 (Denaturation)	25	10
2 (Extension)	50	50
3 (Termination)	85	5

RNase H treatment was then performed to remove RNA, with an incubation of 37ºC for 20 mins. RT-PCR was performed as per section 2.9.3.

4.2.1.2.2 Modified disaggregation protocol

The new disaggregation protocol (see section 2.3.1.1) yielded many more cells for analysis. When cDNA synthesis and RT-PCR was performed as per sections 2.9.2 and 2.9.3, mRNA expression could not be detected; however, utilisation of differing cDNA synthesis and RT-PCR methods was more successful. These latter RT-PCR experiments were performed by Dr. Yvonne Clarkson from Prof. Telfer's lab and were

carried out on bovine tissue only due to low human tissue availability. DDX4-positive cells isolated by Dr. Marie McLaughlin and Dr. Yvonne Clarkson were pooled and analysed. In brief, freshly isolated cells were lysed in TRIzol reagent (Thermo Scientific), cDNA was synthesised using the Omniscript® Reverse Transcription Kit (Qiagen) and RT-PCR was then performed using the *Taq* DNA Polymerase Kit (Qiagen). Primer sequences are detailed in Table 2.4 and 2.5 and were purchased from IDT. Gene fragment template DNA (designed by Dr. Yvonne Clarkson using MacVector7.2 and purchased from IDT) was used as a positive control. RT- samples were used as a negative control to detect contamination.

RT-PCR of freshly isolated cells derived by myself with the modified protocol and using the methods utilised by Dr. Yvonne Clarkson was unsuccessful, therefore the cells were analysed for RNA degradation (see section 4.2.2).

4.2.1.3 Nested RT-PCR of OLCs

Nested RT-PCR is a more sensitive technique than RT-PCR as it involves two rounds of PCR amplification. A different set of primers for the gene of interest is used for each round, with the second set of primers internal to the sequence used in the first round. This both amplifies the signal of the gene of interest and prevents amplification of non-specific products. It was utilised to characterise human OLCs as the number of OLCs was extremely low, with the oocyte-specific gene, *ZP3*, being analysed. The observation of bovine OLCs was so rare that analysis was not possible. Culture medium containing OLCs was aspirated from 2 wells of a 12 well plate containing cultured human cells (Patient 4, Population B, P20), centrifuged at 800 x g for 5 mins and the cell pellet resuspended in RLT buffer containing 1% (v/v) β-ME. Total RNA was extracted as per 2.9.1 and all of the extracted RNA was used for cDNA synthesis. First strand cDNA synthesis was performed using the SuperScript® III First-Strand Synthesis System as per the manufacturer's instructions with subsequent RNase H treatment (see section 4.2.1.2.1). RT-PCR was performed as per section 2.9.3, using the first set of ZP3 primers (Table 4.5) and RPL32 (Table 2.5) as a reference gene. Primers were designed using Primer Blast and purchased from IDT. Ten microlitres

of the samples were run on gels as per section 2.9.3 and the remainder of the first round product was then purified using the QIAquick PCR purification Kit (Qiagen) as per the manufacturer's instructions. The eluted, purified DNA was then used for RT-PCR with the second set of ZP3 primers (Table 4.5) and run on a gel as per section 2.9.3.

Table 4.5. Primer sequences for ZP3 for use in nested PCR experiments.

ZP3 Primer Set	Accession No.	Forward Primer (5'-3')	Reverse Primer (5'-3')	Product size (base pairs)
Set 1	NC_000007	TCCAGGCAGAAATCCACACT	AACAGCAGTCAGAGTCAGGG	623
Set 2		CTTCCATGGCTGTCTTGTCG	GTGCCACAGTCACCTTTGTT	282

4.2.2 Measurement of RNA integrity

Due to problems with detecting mRNA despite the high numbers of cells isolated by myself using the modified dissociation protocol, samples were analysed to assess whether the RNA was being degraded during the sorting process. Analysis of ribosomal RNA (rRNA) was performed as an assessment of the integrity of the less abundant mRNA. Analysis was performed by Dr. Yvonne Clarkson (Prof. Telfer's group) in collaboration with Dr. Jamie McQueen, post-doctoral scientist in Prof. Giles Hardingham's laboratory (Centre of Integrative Physiology, University of Edinburgh). An Agilent 2100 bioanalyser (Agilent Technologies) and the Agilent RNA 6000 Nano Assay Kit (Agilent Technologies) was used to analyse samples on a Agilent RNA 6000 Nano chip (Agilent Technologies) as per the manufacturer's instructions. In brief, the chip was primed with a gel-dye mix before loading dye, a RNA ladder and the sample were loaded onto it. RNA in the samples was then separated by electrophoresis and fluorescence was measured, with the data represented on an electropherogram. Eukaryotic ribosomes are comprised of two rRNA subunits (18S and 28S), and the presence of these subunits was depicted on the graph. The RNA Integrity Number (RIN) was also calculated, with samples of high rRNA integrity having higher RINs

than those of lower quality. A positive control was run simultaneously for comparative purposes.

4.2.3 Immunocytochemistry

Cultured bovine and human cells were characterised for protein expression using ICC as per section 2.7. Bovine cells were fixed in Bouin's and human cells were fixed in 50:50 methanol:ethanol. Normal chicken serum or normal goat serum (both Thermo Scientific) were used depending on the secondary antibody used. The primary and secondary antibodies used are detailed in Tables 4.6 and 4.7. Green tyramide signal amplification and PI nuclear staining was used, with the exception of the mCherry experiments, when blue tyramide signal amplification and Sytox Green nuclear staining was used.

Table 4.6. Primary antibodies used for immunocytochemistry. Antibodies marked by * were used in bovine experiments only; those marked by ** were used in human experiments only. All other antibodies were used for both species. The anti-RFP antibody was used for detection of mCherry.

Primary Antibody	Product Number	Species Raised	Dilution	Source
LIN28	ab46020	Rabbit Polyclonal	1:1000	Abcam
POU5F1*	sc-8628	Goat Polyclonal	1:100	Santa Cruz
IFITM3**	ab74699	Rabbit Polyclonal	1:500	Abcam
C-KIT	A4502	Rabbit Polyclonal	1:100	Dako
DDX4	ab13840	Rabbit Polyclonal	1:300	Abcam
DAZL*	MCA2336	Mouse monoclonal	1:400	AbD Serotec
DAZL**	ab34139	Rabbit polyclonal	1:800	Abcam
RFP**	600-401-379	Rabbit Polyclonal	1:400	Rockland
GFP*	A11122	Rabbit Polyclonal	1:1000	Thermo Scientific

Table 4.7. Secondary antibodies used for immunocytochemistry. Antibody marked by * was used with the primary anti-RFP and anti-GFP antibodies only.

Secondary Antibody	Product Number	Dilution	Source
Chicken anti-rabbit	sc-2963	1:200	Santa Cruz
Chicken anti-mouse	sc-2962	1:100	Santa Cruz
Chicken anti-goat	sc-2953	1:200	Santa Cruz
Goat anti-rabbit*	P1-1000	1:200	Vector

4.2.4 Western blotting

4.2.4.1 Cultured cells

Cultured bovine and human cells were further analysed for protein expression by Western blotting as per section 2.10. For cultured cells, 20-30µg of protein was loaded in each experiment, whilst 10µg of bovine or human fetal ovary protein was used as positive controls. Tables 4.8 and 4.9 detail the primary and secondary antibodies used. Proteins of interest were detected using secondary antibodies visible in the 680nm channel (i.e. red) of the Li-cor Classic infrared imaging system. ACTB was used as a loading control and was detected with a secondary antibody visible in the 800nm channel (i.e. green). The primary antibody was omitted for negative controls.

Table 4.8. Primary antibodies used for Western blotting.

Primary Antibody	Product Number	Species Raised	Dilution	Source
LIN28	ab46020	Rabbit Polyclonal	1:1000	Abcam
IFITM3	ab74699	Rabbit Polyclonal	1:500	Abcam
C-KIT	A4502	Rabbit Polyclonal	1:1500	Dako
DDX4	ab13840	Rabbit Polyclonal	1:500	Abcam
ACTB	A5441	Mouse Monoclonal	1:5000	Sigma-Aldrich

Table 4.9. Secondary antibodies used for Western blotting, with details of fluorescent conjugates.

Secondary Antibody	Conjugate	Product Number	Dilution	Source
Donkey anti-rabbit	Alexa Fluor® 680	A10043	1:10000	Thermo Scientific
Donkey anti-Mouse	IRDye® 800CW	925-32212	1:10000	Li-cor

4.2.4.2 DDX4 detection in freshly isolated and cultured bovine cells

Western blotting of freshly isolated and cultured bovine cells was performed to analyse DDX4 expression by a different methodology in an attempt to improve detection. The experiments were carried out by Dr. Yvonne Clarkson using a standardised protocol utilised in Prof. Telfer's laboratory. Bovine fetal ovary was used as a positive control, while rat skeletal muscle was a negative control. Rat skeletal muscle was selected as a negative control as it was readily available in Prof. Telfer's laboratory and Dr. Clarkson had already validated that the DDX4 antibody could detect the protein in rat. HSP60 was used as a loading control.

4.2.5 Sex chromosomes analysis

To analyse the karyotype of cultured human cells, confluent cells were trypsinised, resuspended in 1ml of OSC culture medium and transported on fresh ice to the South East Scotland Genetics Service (Western General Hospital, Edinburgh). Approximately 30,000 Population B cells from Patient 2 (P7) and 30,000 Patient 8 (P10) were sent to be analysed. A member of staff (Charlotte Keith, Clinical Scientist) performed fluorescence *in situ* hybridisation (FISH) on interphase cells using a XY probe and their standardised protocol to analyse the sex chromosomes.

4.3 Results

4.3.1 Characterisation of freshly isolated bovine cells

4.3.1.1 mRNA expression

During initial experiments using the modified method, cells sorted as being DDX4-positive were pooled so only one population of cells was collected for mRNA analysis (n = 4). These cells were confirmed to express *DDX4* at the mRNA level and also expressed pluripotency markers (*POU5F1* and *LIN28*) and a further marker of the germline (*IFITM3*) (Fig. 4.1). Oocyte-specific markers (*HDAC6*, *GDF9*, *SYCP3*, *ZP3* and *aromatase*) were not expressed by the cells indicating that the DDX4-positive cells were not oocytes. Cells detected as being DDX4-negative by FACS did not demonstrate *DDX4* expression at the mRNA level. These cells did not express *POU5F1* either; however, *LIN28* and *IFITM3* expression was detected, at lower levels than the DDX4-positive cells.

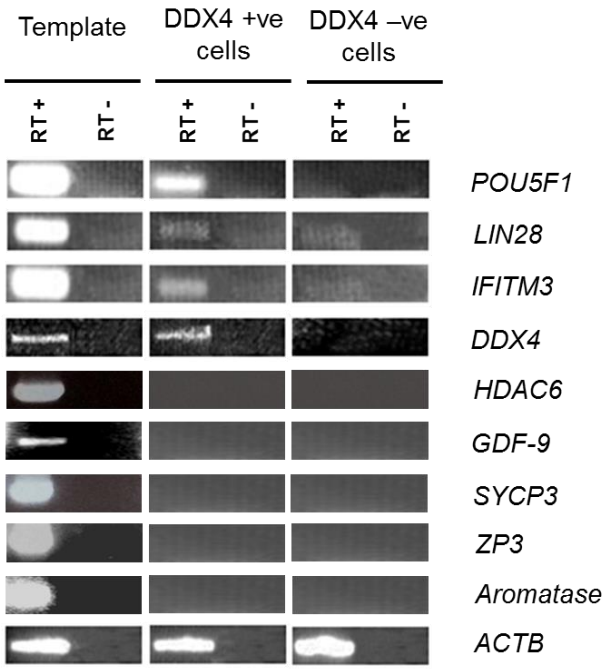


Figure 4.1. RT-PCR results demonstrating the gene expression profile of bovine cells, freshly isolated using the DDX4 antibody. DDX4-positive cells expressed both stem cell and germline markers and did not express oocyte-specific markers. *β-actin* (*ACTB*) was used as a reference gene. Gene fragment template DNA was used as the positive control.

Subsequent experiments utilising the modified protocol did detect Populations A and B and yielded large numbers of cells, but no detection of any mRNA, including the reference gene, was demonstrated on RT-PCR because of RNA degradation (Fig. 4.2). Due to time limitations, further isolation using this method was not performed.

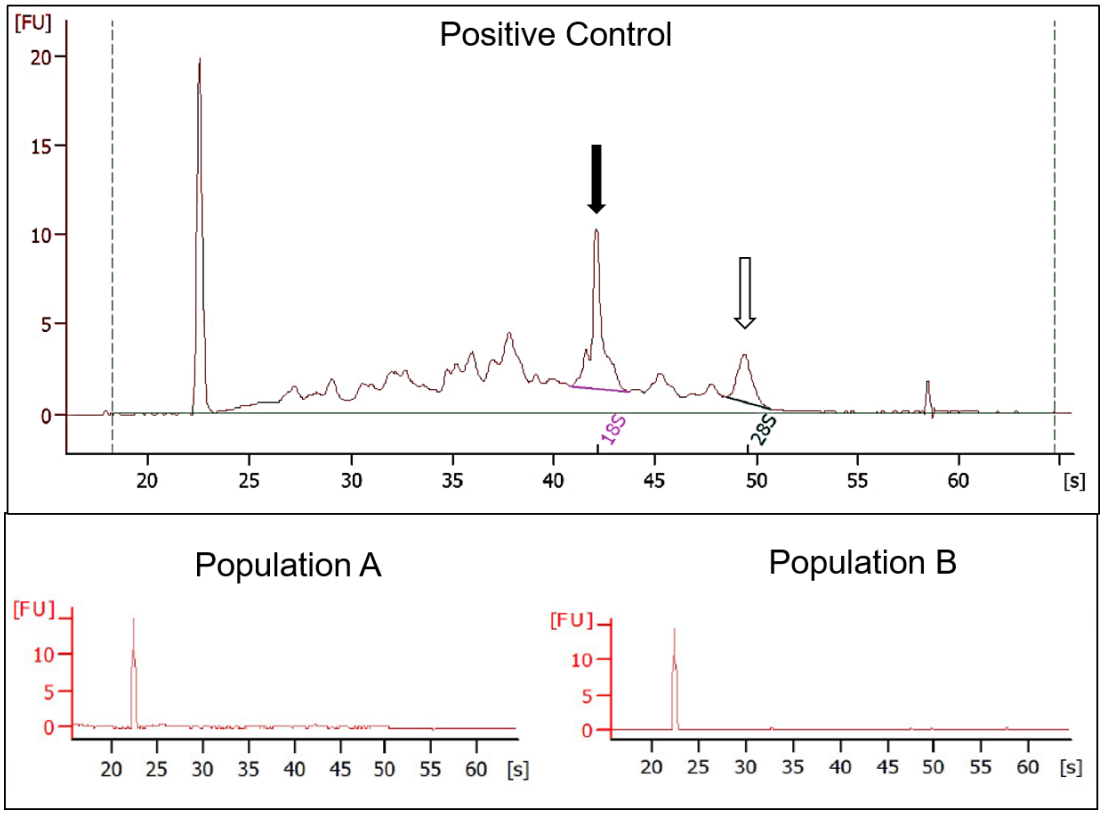


Figure 4.2. Electropherograms demonstrating RNA integrity of RNA from freshly isolated bovine cells. The positive control demonstrates detected peaks corresponding to the 18S (black arrow) and 28S (white arrow) ribosomal subunits (RIN: 6.5). These peaks were not detected in either Population A or B cells and the RIN numbers for both samples were undetectable indicating that the RNA was degraded.

4.3.1.2 Protein expression

Freshly isolated cells derived by Dr. Marie McLaughlin and Dr. Yvonne Clarkson were also collected for protein analysis by Western blot (n = 1). Cells isolated as DDX4-positive by FACS expressed DDX4 faintly at the protein level, whilst cells detected as being DDX4-negative by FACS did not demonstrate DDX4 protein expression (Fig. 4.3).

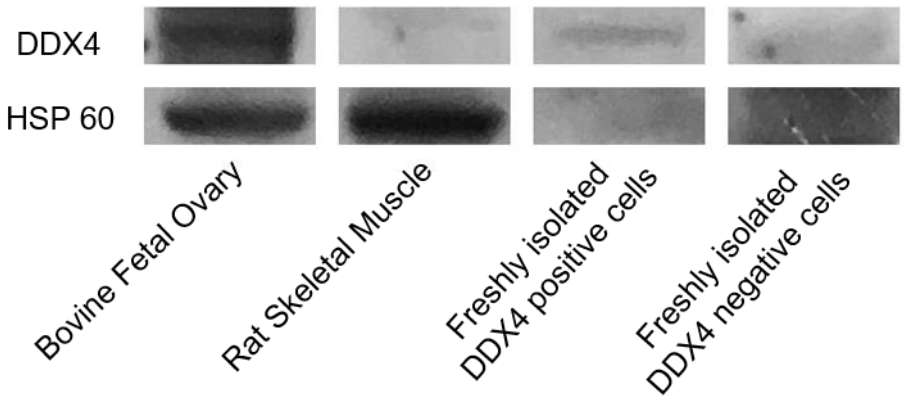


Figure 4.3. Western blot analysis of freshly isolated bovine cells demonstrating DDX4 expression (expected molecular weight of 76 kDa) in cells selected as DDX4-positive. Bovine fetal ovary was used as a positive control and rat skeletal muscle was used as a negative control. HSP60 was used as a loading control (n=1). The low levels of HSP60 expression in the freshly isolated cells reflects the reduced protein concentrations in comparison to the positive and negative controls.

4.3.2 Characterisation of cultured bovine cells

4.3.2.1 mRNA expression

The expression of both pluripotency (*POU5F1* and *LIN28*) and germline (*IFITM3*, *PRDM1*) markers was demonstrated in cultured Population B bovine cells of all cell lines from the first passage onwards (Fig. 4.4 and Table 4.10). Cultured bovine cells also consistently expressed the oocyte marker, *NOBOX*. This molecular signature was consistent over long-term *in vitro* culture. In contrast, *C-KIT* was not detected in initial

passages but was consistently detected after P14. *DDX4* expression was detected once during *in vitro* culture, whilst *DPPA3* expression was never detected. The single Population A cell line exhibited an identical gene expression pattern to early passage cells of Population B. Bovine *NANOG* primers were designed but could not be validated for use as no band was detected in the positive control. Of note, cryopreservation did not affect the pattern of gene expression.

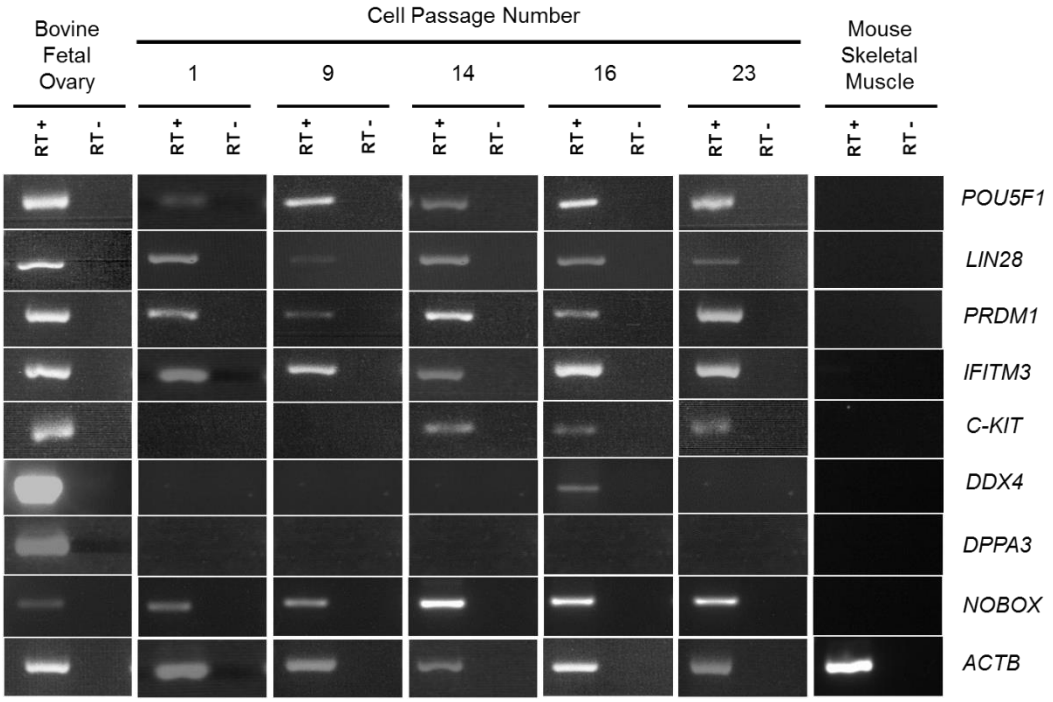


Figure 4.4. RT-PCR results of cultured Cell Line 4, Population B, bovine cells demonstrating expression of stem cell, germline and oocyte markers across passages. The results are representative of the other bovine cell lines. *POU5F1*, *LIN28*, *PRDM1*, *IFITM3* and *NOBOX* expression was consistent, whereas *C-KIT* and *DDX4* expression was variable and cells did not express *DPPA3*. *β-actin* (*ACTB*) was used as a reference gene. Bovine fetal ovary was used as the positive control and mouse skeletal muscle was used as a negative control.

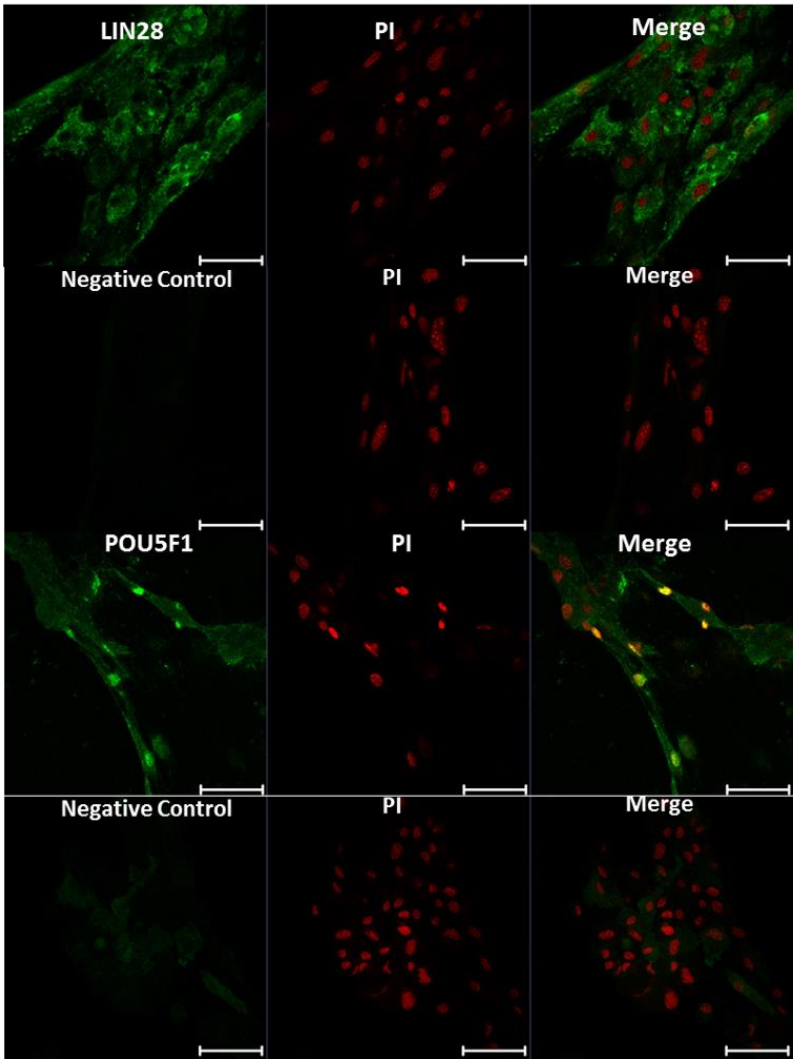
Table 4.10. Comparison of mRNA expression of stem cell, germline and oocyte markers between bovine cells lines of varying passages. Consistent expression of *POU5F1*, *LIN28*, *PRDM1*, *IFITM3* and *NOBOX* and lack of detection of *DPPA3* was observed in the cultured cells; *C-KIT* and *DDX4* expression was variable and only seen in later passages. Grey boxes illustrate markers that were not examined in the freshly isolated cells (experiments performed by a colleague). * *NOBOX* was not examined in freshly isolated cells, however, other oocyte markers (e.g. *GDF-9*, *ZP3*) were investigated and were not detected.

Cell Line	Passages	*POU5F1*	*LIN28*	*PRDM1*	*IFITM3*	*C-KIT*	*DDX4*	*DPPA3*	*NOBOX*
Freshly isolated	N/A	✓	✓		✓		✓		*
1 (Pop. B)	1, 3, 4	✓	✓	✓	✓	X	X	X	✓
2 (Pop. B)	5, 10	✓	✓	✓	✓	X	X	X	✓
3 (Pop. B)	4, 7	✓	✓	✓	✓	X	X	X	✓
4 (Pop. B)	1, 9	✓	✓	✓	✓	X	X	X	✓
	14, 23	✓	✓	✓	✓	✓	X	X	✓
	16	✓	✓	✓	✓	✓	✓	X	✓
5 (Pop. B)	3, 7	✓	✓	✓	✓	X	X	X	✓
6 (Pop. A)	4, 5	✓	✓	✓	✓	X	X	X	✓

4.3.2.2 Protein expression

Protein expression of cultured bovine cells was analysed by both ICC and Western blotting. Immunocytochemical analysis demonstrated that the cells expressed both pluripotency (LIN28 and POU5F1) and germline (C-KIT, DDX4 and DAZL) markers, with POU5F1 expression being detected in the nucleus, as expected, and the other markers being located in the cytoplasm (Fig. 4.5). Expression of each protein was detected in all analysed cells. In addition, GFP expression could be detected in some cells exposed to GFP-containing lentivirus. Optimal images were achieved using Bouin's fixed cells and a permeabilisation time of 20 mins.

A


LIN28
PI
Merge
Negative Control
PI
Merge
POU5F1
PI
Merge
Negative Control
PI
Merge

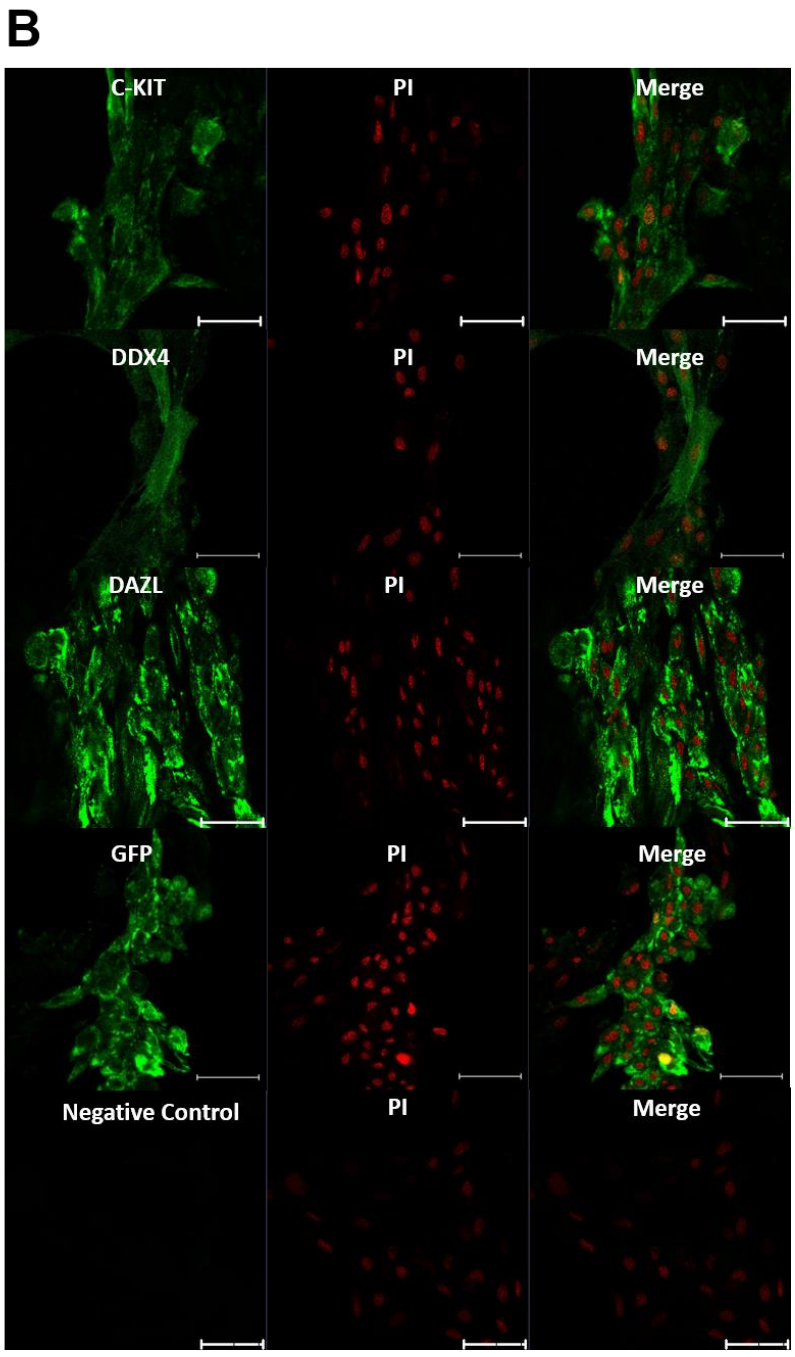


Figure 4.5. Immunocytochemistry of cultured bovine cells demonstrating expression of (A) pluripotency (LIN28 and POU5F1) and (B) germ cell (C-KIT, DDX4 and DAZL) markers. GFP-lentiviral transduction resulted in a proportion of cells expressing the GFP protein. Propidium iodide (PI) was used as a nuclear counterstain. The images are of Cell Line 4, Population B, P13 cells. Negative controls (primary antibodies omitted) are shown for comparison. Scale bars = 50µm.

Western blotting demonstrated IFITM3 protein expression in cultured bovine cells (Fig. 4.6). LIN28 and C-KIT expression was not detected. DDX4 expression could not be detected in the positive control (bovine fetal ovary) initially, although several other bands were seen, corresponding to different molecular weights (Fig. 4.7(a)). However, when an altered Western blot methodology was used, a band of the predicted size for DDX4 (76kDa) was detected consistently in the bovine fetal ovary. Expression in cultured bovine cells was more variable (Fig. 4.7(b)).

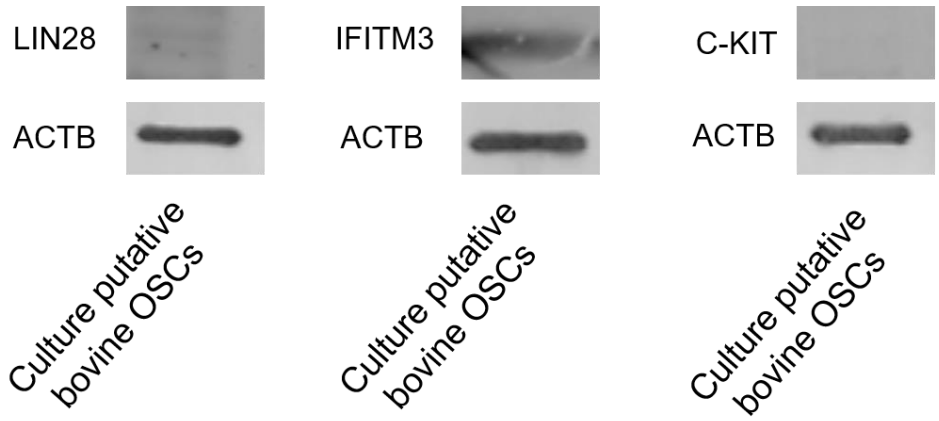


Figure 4.6. Western blot analysis of cultured bovine cells demonstrating IFITM3 expression (expected molecular weight (mw) of 15 kDa), but no LIN28 (29 kDa) or C-KIT (145 kDa) expression. Samples were also probed for β-actin (ACTB) as a reference gene (expected mw 42 kDa). The images are of Cell Line 4, Population B, P16.

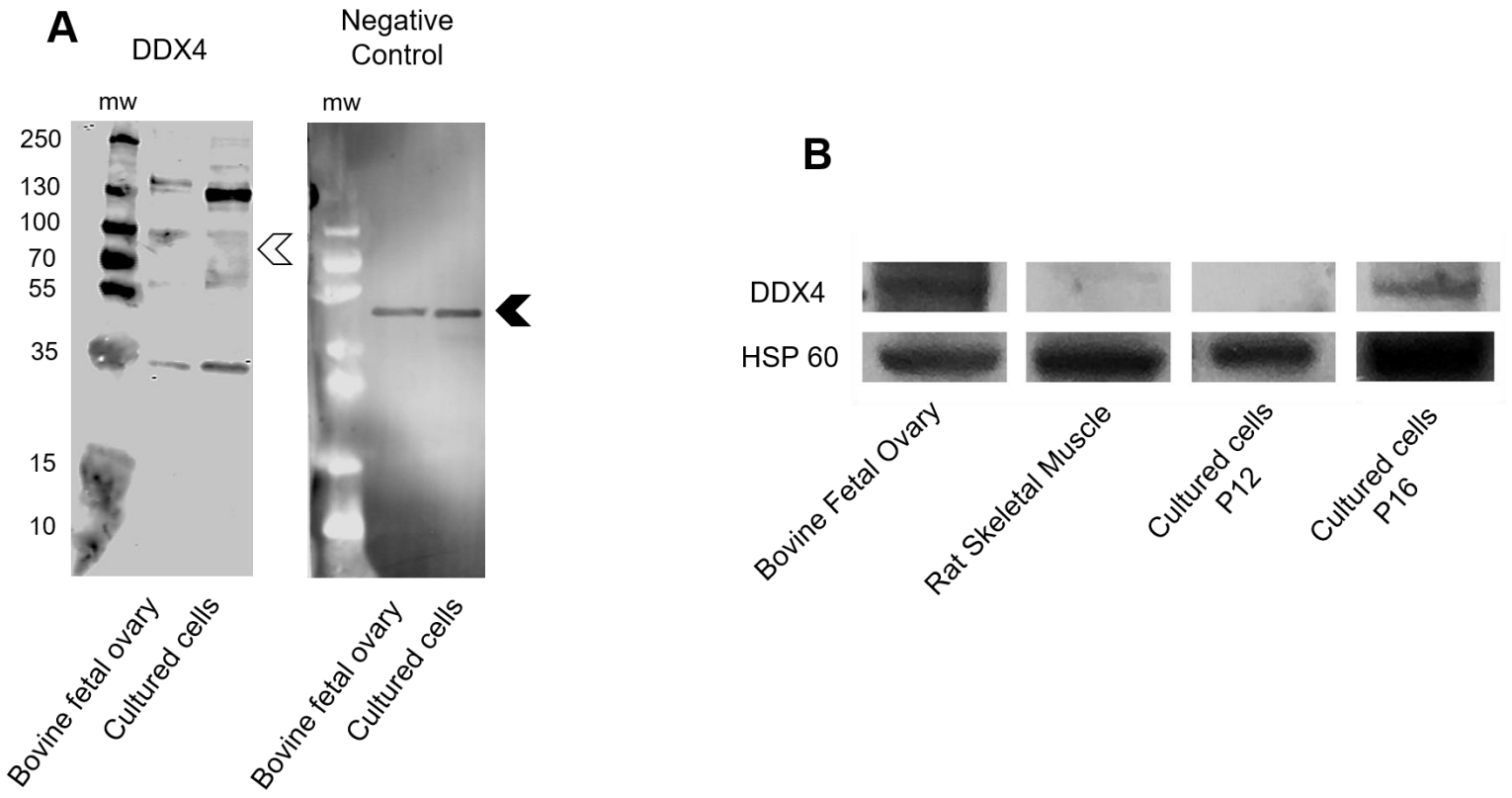


Figure 4.7. Western blot analysis for DDX4 expression. **(A)** Probing of bovine fetal ovary and cultured bovine cells for DDX4 with the original methodology failed to detect the protein (white arrow; expected mw 76 kDa) but did detect several other bands, which were not present when the primary antibody was omitted (negative control: black arrow denotes bands for the reference gene, ACTB, to show that protein was loaded). **(B)** Using an altered methodology (see section 4.2.4.2), a colleague (Dr. Clarkson) did detect DDX4 using the same primary antibody in bovine fetal ovary (positive control); however, expression in cultured bovine cells was variable. Rat skeletal muscle was used as a negative control. HSP60 was used as a loading control. (Cell line 4, Population B, P12 and P16).

4.3.3 Characterisation of human cells

4.3.3.1 mRNA expression

Analysis of freshly isolated human cells was attempted once, with no mRNA expression of even the reference gene detected. Due to scarcity of tissue, further experiments were not performed.

Cultured Population B human cells expressed both pluripotency (*POU5F1*, *LIN28* and *NANOG)* and germ cell *(IFITM3*, *DPPA3*, *PRDM1* and *C-KIT)* markers consistently across passages (Fig. 4.8 and Table 4.11). The oocyte-specific marker, *GDF-9*, was also consistently expressed. *DDX4* mRNA expression was again inconsistent, being detected in 3 out of the 5 patients and only after passage 10. Population A cells from Patient 8 had an identical molecular signature to Population B cells; however, Patient 4's Population A cells demonstrated more inconsistent expression, with *NANOG*, *IFITM3* and *PRDM1* detected in passage 4 cells and *NANOG*, *POU5F1*, *CKIT* and *DPPA3* detected in passage 7 cells. Neither cryopreservation nor lentiviral transduction altered the gene expression pattern of the cells.

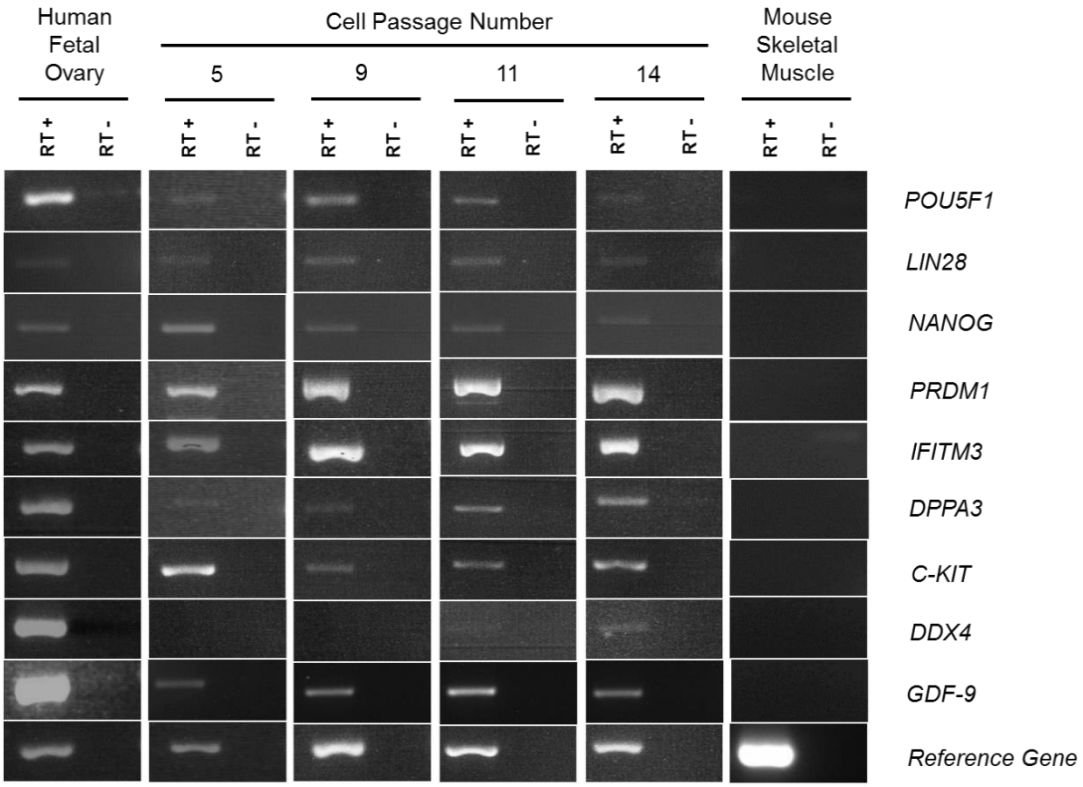


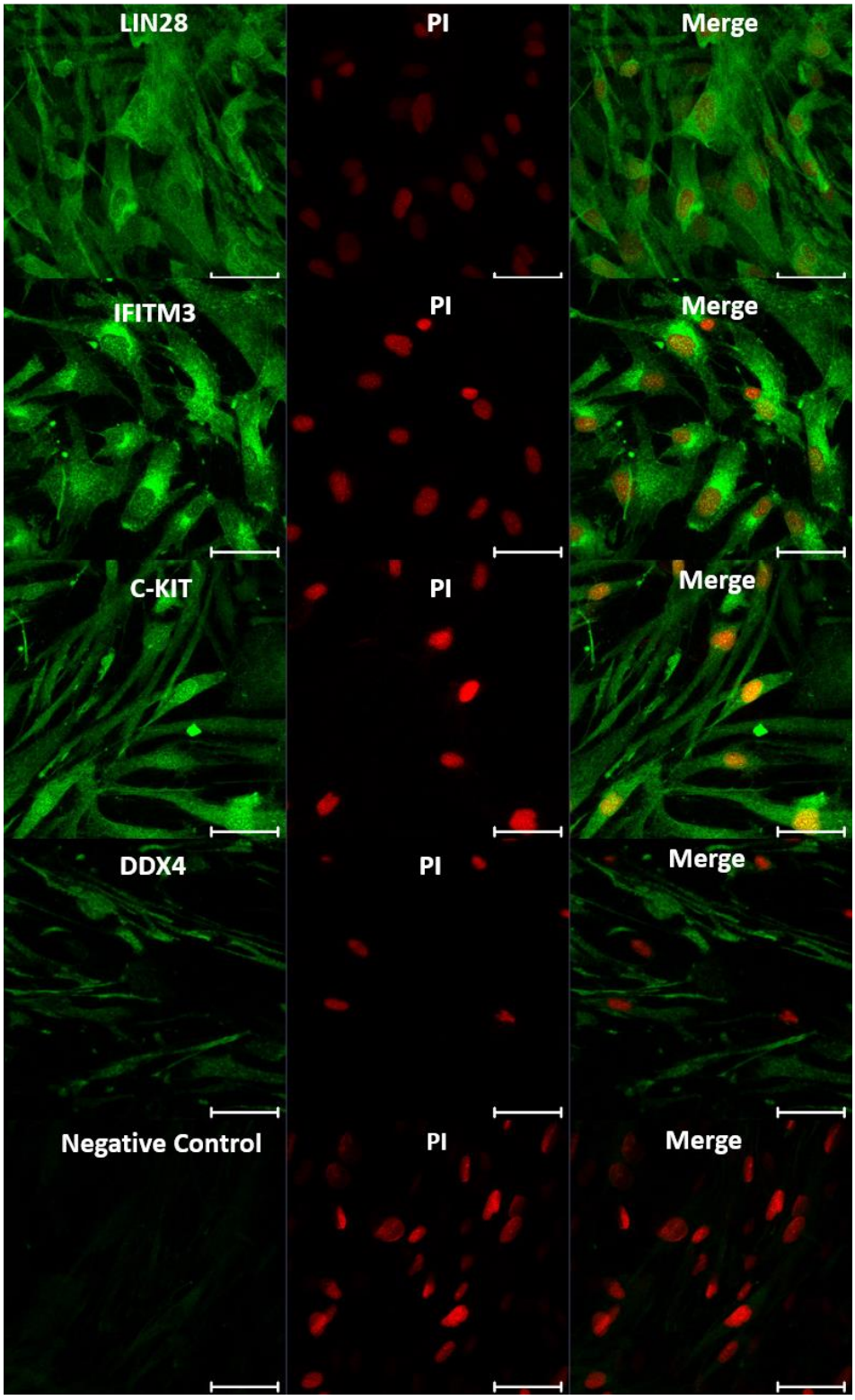
Figure 4.8. RT-PCR results of cultured Patient 4, Population B, human cells demonstrating expression of stem cell, germline and oocyte markers across passages. The results are representative of the other human cell lines. *POU5F1*, *LIN28*, *NANOG*, *PRDM1*, *IFITM3*, *DPPA3*, *C-KIT* and *GDF-9* expression was consistent, whereas *DDX4* expression was variable. Human fetal ovary was used as the positive control and mouse skeletal muscle was used as a negative control. *RPL32* was used as a reference gene for human tissue, whilst *β-actin* was used for mouse tissue.

Table 4.11. Comparison of mRNA expression of stem cell, germline and oocyte markers between human cells lines of varying passages. Consistent expression of *POU5F1*, *LIN28*, *NANOG*, *PRDM1*, *IFITM3*, *DPPA3*, *C-KIT* and *GDF-9* was observed in the cultured Population B and Patient 8's Population A cells. *DDX4* expression was variable and only seen in later passages in 3 patients. * Technical issues with the columns used in RNA extraction resulted in extremely low levels of RNA, therefore lack of detection of some markers was possibly due to low RNA concentrations rather than lack of expression by these cells.

Cell Line	Passage	*POU5F1*	*LIN28*	*NANOG*	*PRDM1*	*IFITM3*	*DPPA3*	*C-KIT*	*DDX4*	*GDF-9*
1 (Pop. B)	1, 2, 10	✓	✓	✓	✓	✓	✓	✓	X	✓
	6	*	*	*	✓	✓	*	*	*	*
2 (Pop. B)	2, 4, 9, 13	✓	✓	✓	✓	✓	✓	✓	X	✓
	11	✓	✓	✓	✓	✓	✓	✓	✓	✓
3 (Pop. B)	1, 2	✓	✓	✓	✓	✓	✓	✓	X	✓
4 (Pop. A)	4	X	X	✓	✓	✓	X	X	X	✓
	7	✓	X	✓	X	X	✓	✓	X	✓
4 (Pop. B)	5, 9	✓	✓	✓	✓	✓	✓	✓	X	✓
	11	✓	✓	✓	✓	✓	✓	✓	✓	✓
	14	✓	✓	✓	✓	✓	✓	✓	✓	✓
8 (Pop. A)	1, 3, 5, 16	✓	✓	✓	✓	✓	✓	✓	X	✓
	10	✓	✓	✓	✓	✓	✓	✓	✓	✓
8 (Pop. B)	1, 3, 5, 11	✓	✓	✓	✓	✓	✓	✓	X	✓

4.3.3.2 Protein expression

Cultured human cells demonstrated immunocytochemical protein expression of LIN28, IFITM3, C-KIT, DDX4 and DAZL (Fig. 4.9). As seen in the bovine cells, expression appeared to be ubiquitous in the cell samples investigated. Cells exposed to mCherry-containing lentivirus demonstrated mCherry protein expression. Optimal images were achieved using 50:50 methanol:ethanol as a fixative.


LIN28
PI
Merge
IFITM3
PI
Merge
C-KIT
PI
Merge
DDX4
PI
Merge
Negative Control
PI
Merge

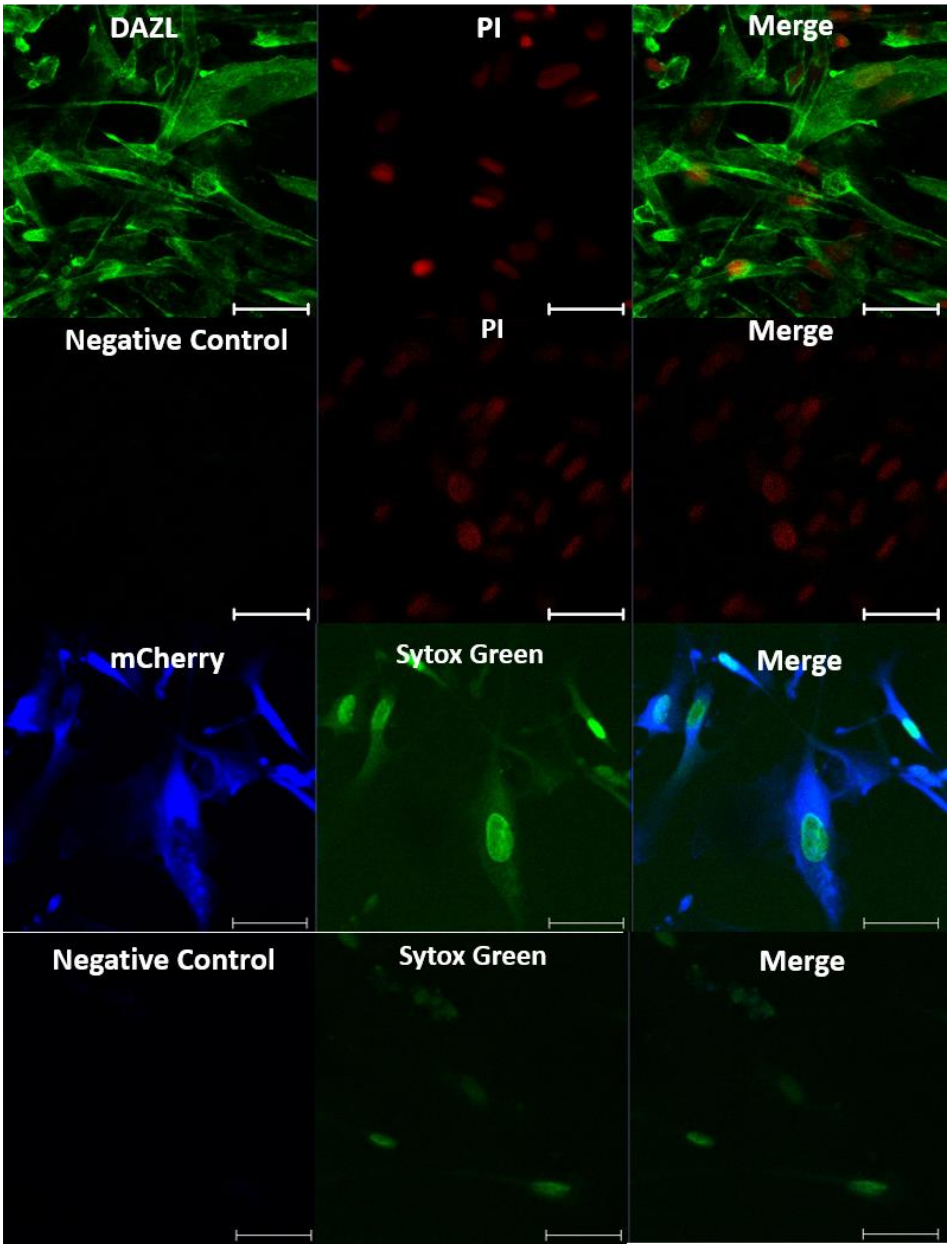
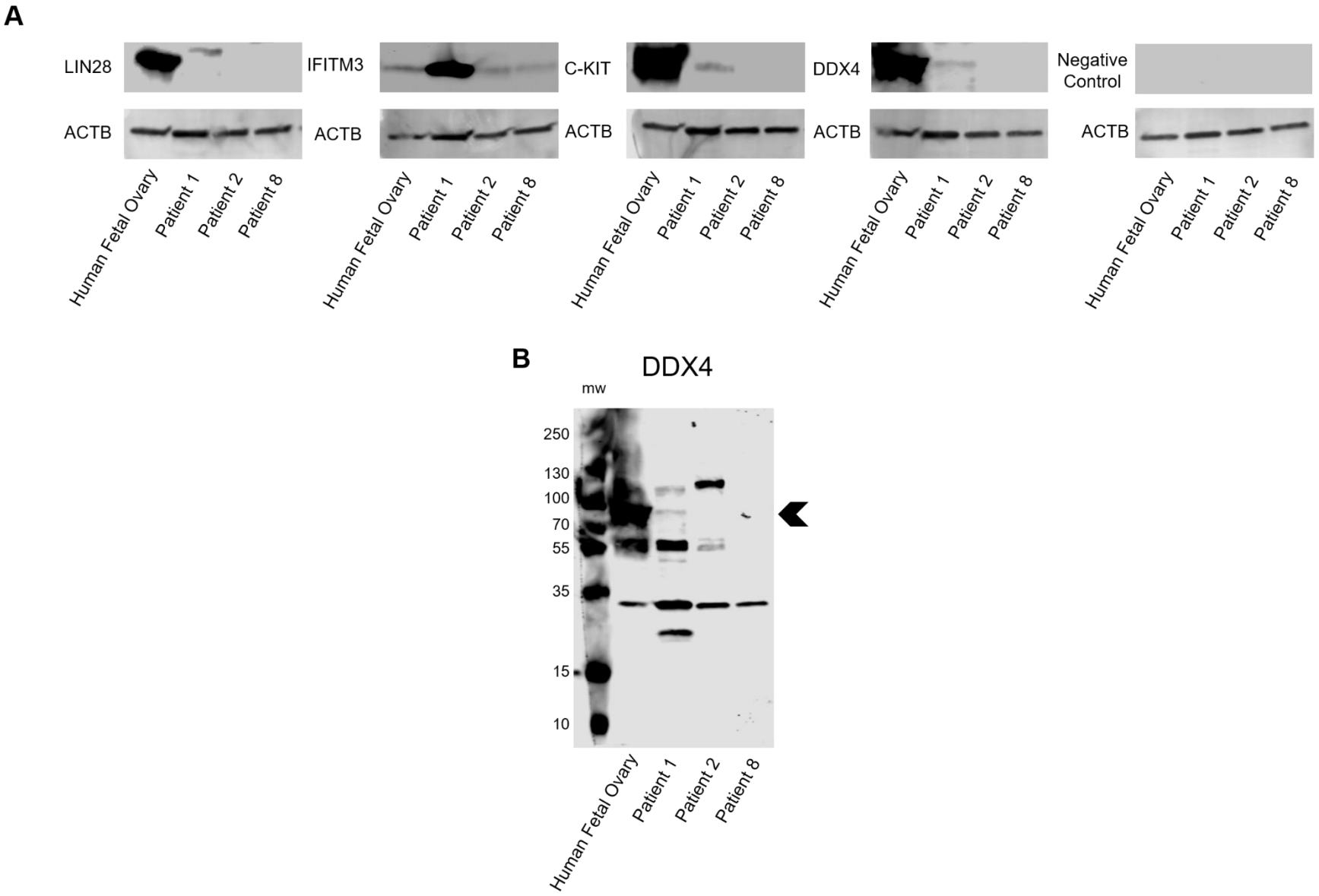


Figure 4.9. Immunocytochemistry of cultured human cells demonstrating expression of pluripotency (LIN28) and germ cell (IFITM3, C-KIT, DDX4 and DAZL) markers. Propidium iodide (PI) was used as a nuclear counterstain. The images are of Patient 1, Population B, P13 cells. Transduction with mCherry-lentiviruses resulted in some cells expressing the mCherry protein. Sytox green was used as a nuclear counterstain for these cells. The images are of Patient 1, Population B, P15 cells. Negative controls (primary antibodies omitted) are shown for comparison. Scale bars = 50µm.

Western blotting for IFITM3, C-KIT and DDX4 confirmed that the cells express these germline markers at the protein level (Fig. 4.10), although C-KIT and DDX4 expression was variable. LIN28 expression was not demonstrated. DDX4 detection was improved when an alternative protocol was used, but variable expression was still demonstrated (Fig. 4.11). Again, bands corresponding to other molecular weights were observed (Fig. 4.10(b)).

Figure 4.10. Western blot analysis of cultured human cells from different patients for pluripotency and germline markers. (A) Cells demonstrated IFITM3 expression consistently (expected mw 15 kDa), whilst C-KIT (145 kDa) and DDX4 (76 kDa) expression was variable. LIN28 (29 kDa) was not detected. Samples were also probed for β-actin as a reference gene (expected mw 42 kDa). Human fetal ovary was used as a positive control and the primary antibodies were omitted for the negative control. **(B)** When the cells were probed for DDX4, multiple bands corresponding to other molecular weights were observed in addition to the band at the expected mw of 76 kDa (black arrow). Patient 1 cells = Population B, P11, Patient 2 cells = Population B, P14, Patient 8 cells = Population A, P16.


A
LIN28
ACTB
IFITM3
ACTB
C-KIT
ACTB
DDX4
ACTB
Negative Control
ACTB
Human Fetal Ovary
Patient 1
Patient 2
Patient 8
B
DDX4
mw
250
130
100
70
55
35
15
10
Human Fetal Ovary
Patient 1
Patient 2
Patient 8

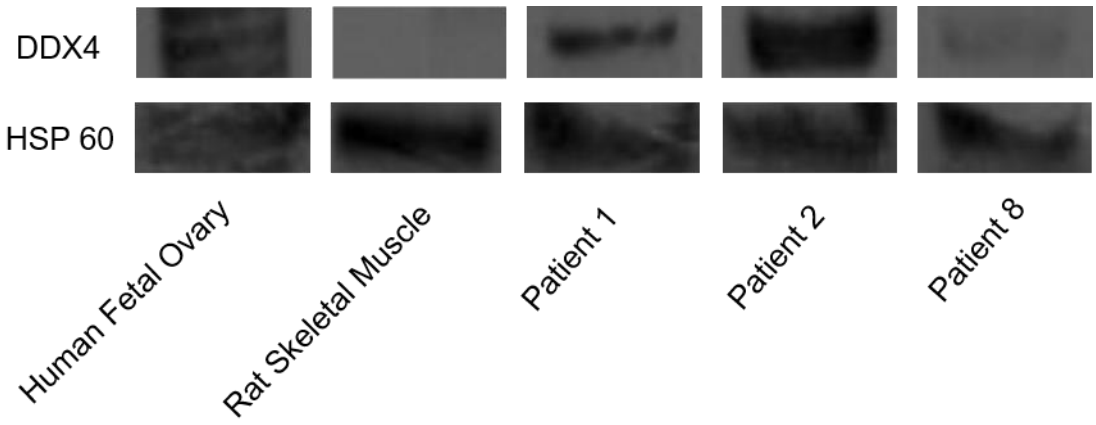


Figure 4.11. Using a different Western blotting methodology, variable expression of DDX4 could be detected in cultured human cells. Human fetal ovary was used as a positive control and rat skeletal muscle was a negative control. HSP60 was a loading control. Patient 1 cells = Population B, P11, Patient 2 cells = Population B, P14, Patient 8 cells = Population A, P16.

4.3.3.3 Sex Chromosome Analysis

A full karyotype of the cultured human cells was not possible as none were in metaphase. FISH analysis of cells in interphase from two patients demonstrated that the cells contained two copies of the X chromosome (Fig. 4.12). The total number of cells examined by FISH was not provided by the Clinical Scientist performing the analysis, however, all analysed cells were reported to be XX.

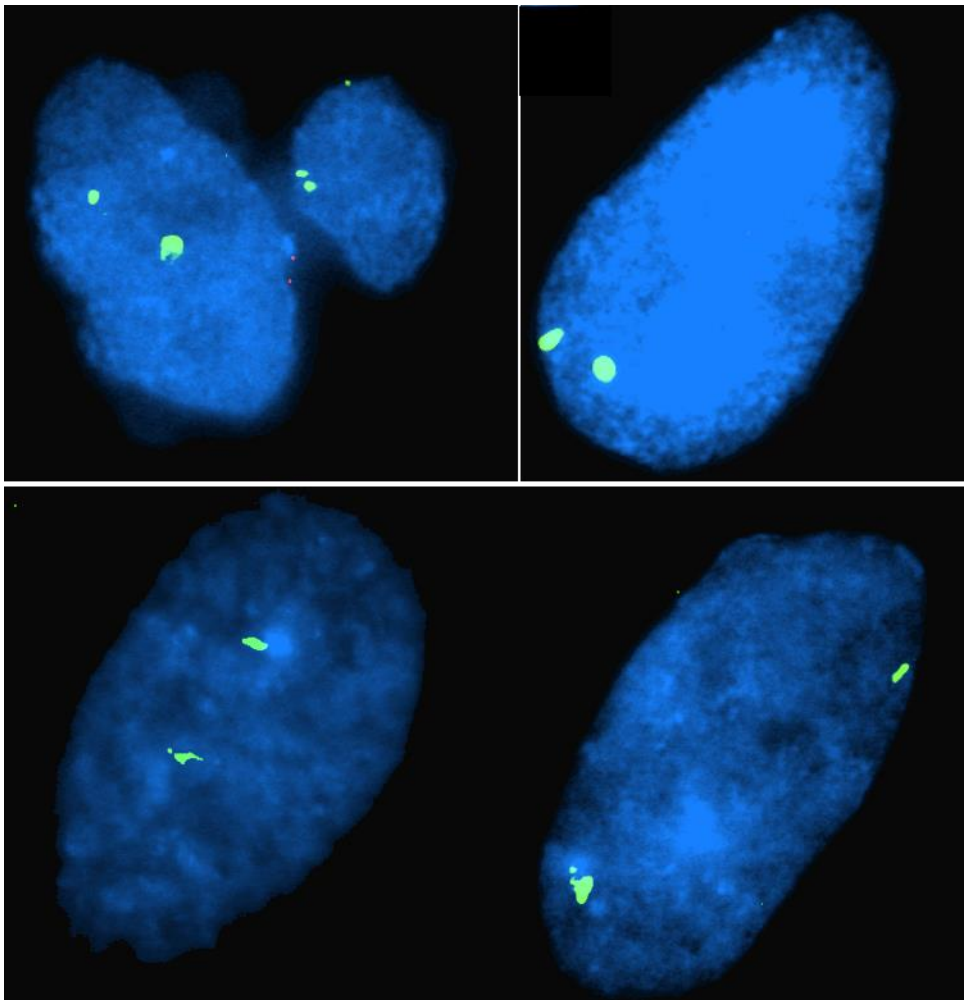


Figure 4.12. FISH analysis of cultured Population B human cells demonstrated that the cells were XX. The green areas represent the X chromosomes, with each cell containing X chromosomes. DAPI (blue) was used as the counterstain.

4.3.4 Characterisation of human OLCs

Nested RT-PCR demonstrated that non-adherent human cells in *in vitro* culture (a small number of which were OLCs) did not express the oocyte-specific marker, *ZP3* (Fig. 4.13).

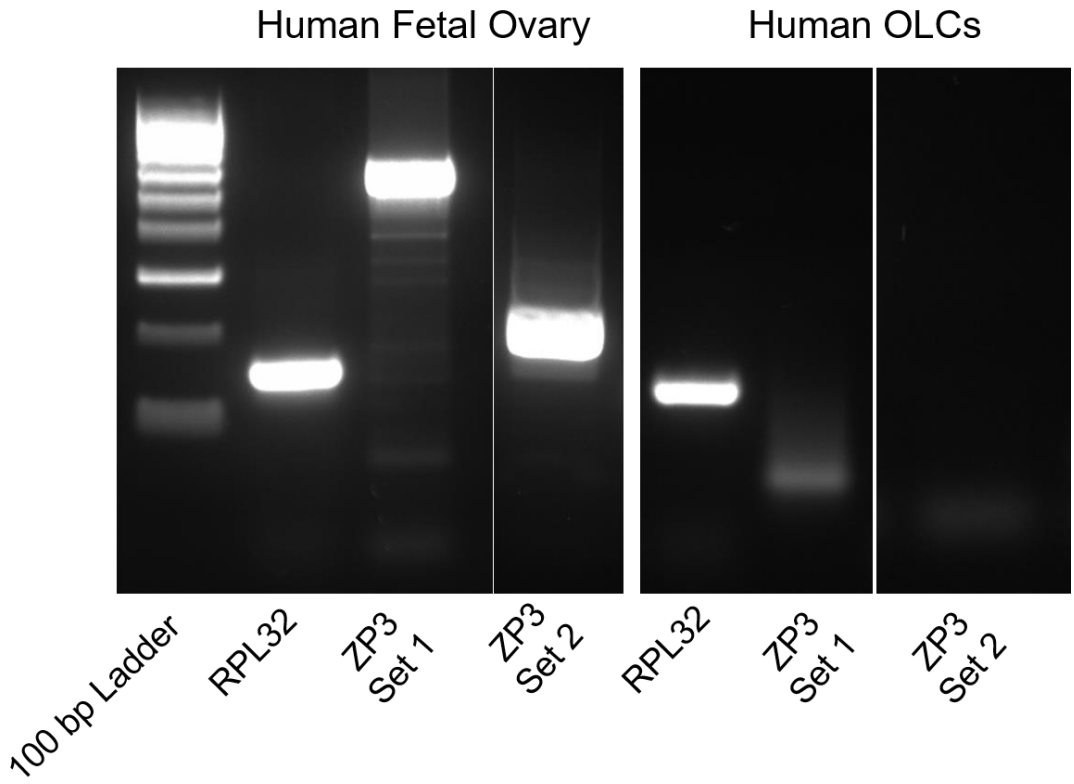


Figure 4.13. Nested RT-PCR demonstrating that non-adherent cells in *in vitro* culture (Patient 1, Population B, P20) did not express the oocyte-specific gene, *ZP3*. Human fetal ovary was used as a positive control and demonstrated expression of both the reference gene (*RPL32*) and the two successive *ZP3* primers. Although the OLCs expressed *RPL32*, no *ZP3* expression was detected. bp = base pair.

4.4 Discussion

4.4.1 The molecular signature of isolated cells

The results of this Chapter indicate that the cells isolated from adult bovine and human ovarian cortex have the molecular characteristics expected of an OSC, with expression of both pluripotency and germline markers detected. Furthermore, human cells contain two copies of the X chromosome, suggesting the cells are diploid. This is the ploidy of all cells with the exception of oocytes just after fertilisation (following extrusion of the second polar body) and spermatozoa, which are haploid. The gene expression pattern of freshly isolated bovine cells corroborates that of previous studies, with the detection of *POU5F1* (Pacchiarotti *et al.*, 2010), *IFITM3* (White *et al.*, 2012) and *DDX4* (Pacchiarotti *et al.*, 2010, White *et al.*, 2012) as well as *LIN28* (which, to date, has only been analysed at the protein level in cultured cells (Hu *et al.*, 2012)). Moreover, the finding that freshly isolated cells express DDX4 at both the mRNA and protein level supports the use of anti-DDX4 antibodies in selecting for putative OSCs.

Cultured bovine cells demonstrated consistent LIN28, POU5F1 and IFITM3 expression at the mRNA and protein level, plus *PRDM1* mRNA expression and DAZL protein expression. Cultured human cells expressed both the mRNA and protein of LIN28, IFITM3 and C-KIT, the mRNA of *POU5F1*, *NANOG*, *PRDM1* and *DPPA3* and the DAZL protein consistently. This is the first time that protein expression of putative OSC markers (namely IFITM3, C-KIT, DDX4) has been demonstrated by the use of Western blotting, which confirms the expression seen on ICC. This pattern of gene expression is in keeping with the previous findings in the literature (Zou *et al.*, 2009, Pacchiarotti *et al.*, 2010, Zou *et al.*, 2011, Hu *et al.*, 2012, White *et al.*, 2012, Wolff *et al.*, 2013, Wolff *et al.*, 2014, Bui *et al.*, 2014, Zhou *et al.*, 2014, Hernandez *et al.*, 2015, Xiong *et al.*, 2015, Lu *et al.*, 2016). The detection of *NANOG* supports the results reported by others (Pacchiarotti *et al.*, 2010, Hu *et al.*, 2012, Bui *et al.*, 2014, Hernandez *et al.*, 2015) but contradicts the data reported by Wu's group (Zou *et al.*, 2009, Zou *et al.*, 2011, Zhou *et al.*, 2014).

It is interesting to note, however, that DDX4 expression in cultured cells from both species is variable and infrequent, both at the mRNA and protein level, indicating that it may be both down- and up-regulated during *in vitro* culture. This inconsistency in expression may explain why others have been unable to identify *DDX4* in cultured cells (Hernandez *et al.*, 2015). Although the general pattern of mRNA expression over

time is otherwise very consistent, cultured bovine cells also have fluctuating *C-KIT* expression: it appears that the cells acquire the ability to express this germline marker over time. Although the speed at which the cells established in culture was postulated to lower the chance of *in vitro* transformation, characterisation of the cells indicates that molecular changes are indeed occurring *in vitro*. This phenomenon may be due to differentiation of some of the cells and may reflect the changes that occur during normal human germ cell development, where increased DDX4 expression occurs simultaneously with downregulation of pluripotency markers (e.g. POU5F1) (Stoop *et al.*, 2005, Anderson *et al.*, 2007). Moreover, recent mouse research has led to the hypothesis that small POU5F1-expressing "germ stem cells" in adult ovaries can enter a developmental schedule such that cells lose their ability to express POU5F1 whilst sequentially acquiring the ability to express the germline markers DAZL and DDX4, before undergoing meiosis and folliculogenesis (Guo *et al.*, 2016). However, other explanations are also possible: firstly, differentiation of the cells *in vitro* could be a result of removal from their stem cell niche which may have been maintaining the putative OSCs in a less differentiated state (as seen in *C. elegans* and *Drosophila*; see section 1.2.3). Secondly, the cells' gene expression could be caused by prolonged *in vitro* culture and passaging. Finally, as *C-KIT* was only faintly detected in cultured bovine cells when more cells (and thus RNA) were available for analysis, perhaps at the earlier passages there was inadequate RNA for *C-KIT* expression to be perceived.

The gene expression pattern of Population A and B cells was identical, with the exception of Patient 4, where markers were more inconsistently expressed in Population A. Interestingly, this was also the cell population that didn't grow as readily as its Population B counterpart. Therefore, although it appears, by morphology, growth and gene expression, that Population A is identical to Population B, Patient 4's Population A is an exception, the reason for which has not yet been elucidated.

With regards interspecies variation, although the molecular signature of cultured cells from both species was very similar, there was a notable difference in mRNA expression for one germline marker: *DPPA3*. The reason for this unclear as it has been detected in mouse (Zou *et al.*, 2009, White *et al.*, 2012, Hernandez *et al.*, 2015), pig (Bui *et al.*, 2014), monkey (Wolff *et al.*, 2013, Wolff *et al.*, 2014) and human (White

et al., 2012, Hernandez *et al.*, 2015) OSCs at the mRNA and/or protein level previously, therefore one would expect it to be present in bovine cells.

Lastly with regards characterisation, lentiviral-transduced cells demonstrated protein expression of GFP (bovine cells) and mCherry (human cells), verifying that the fluorescence observed is due to the cells translating the fluorescent proteins.

4.4.2 *In vitro neo*-oogenesis

The analysis of oocyte-specific markers was performed in both freshly isolated and cultured bovine cells. Several oocyte-specific markers were not detected in freshly isolated cells, indicating that this cell population was not contaminated with oocytes. Therefore, the isolated DDX4-positive cells must be a distinct population from oocytes. In *in vitro* culture, however, *NOBOX*, a marker of oocytes in both primordial and growing follicles (Suzumori *et al.*, 2002) with proven function in cows (Tripurani *et al.*, 2011), was detected. Correspondingly, cultured human cells expressed *GDF-9*, a member of the TGFβ family which has been found to be expressed in oocytes within the human fetal ovary prior to follicle formation (Bayne *et al.*, 2015). These results are in keeping with those of White *et al.*, who reported that oocyte markers were absent in freshly isolated cells but became detectable during *in vitro* culture (White *et al.*, 2012). This finding suggests that some cells may be differentiating into oocytes spontaneously and, indeed, OLCs were observed infrequently floating in the culture medium of both bovine and human cells, as seen previously in mice (Pacchiarotti *et al.*, 2010, White *et al.*, 2012), pigs (Bui *et al.*, 2014) and humans (Hernandez *et al.*, 2015, White *et al.*, 2012). However, analysis of non-adherent cells for ZP3 (one of four glycoproteins which comprise the zona pellucida surrounding the human oocyte (Lefievre *et al.*, 2004)), did not detect any expression. Thus, although the adherent cells showed molecular evidence of undergoing spontaneous differentiation into oocytes, the non-adherent OLCs did not appear to be the product of this transformation. It is possible that these OLCs were in fact degenerating cells which were detaching from the culture plate surface. This finding is in contrast to the results of other groups, where the authors report that the non-adherent OLCs do express oocyte-specific markers and thus conclude that spontaneous *neo*-oogenesis is occurring (Pacchiarotti *et al.*, 2010, White *et al.*, 2012).

4.4.3 Summary

In conclusion to this Chapter, the cells isolated on the basis of DDX4 from adult bovine and human ovarian cortex possess a molecular signature that indicates they are putative OSCs, with dual expression of pluripotency and germline markers demonstrating their early germline nature. Moreover, the results detailed in this Chapter comprise one of the most comprehensive characterisation analysis in the OSC literature to date. The DDX4-positive cells detected by flow cytometry are not oocytes, yet oocyte markers are expressed by the cells once they are cultured *in vitro*. This fact, coupled with the variations in *DDX4* and *C-KIT* expression indicates that the cells may be undergoing *in vitro* transformation or differentiation. However, the OLCs observed in culture do not express a classic oocyte marker, therefore the cells' potential to undergo spontaneous *in vitro neo*-oogenesis has not been confirmed. If these cells are to be definitively regarded as OSCs, their functional capabilities must thus be tested, which is the focus of Chapter 5.

Chapter 5

The *In Vitro Neo*-oogenesis Potential of Putative OSCs

5.1 Introduction

5.1.1 OSCs and *Neo*-oogenesis

The definitive test of a putative OSC is to demonstrate that the cell has the capability to differentiate into a developmentally competent and fertilisable oocyte. To date, both *in vitro* and *in vivo* approaches have been utilised by investigators to verify that they have isolated OSCs (Zou *et al.*, 2009, Zou *et al.*, 2011, White *et al.*, 2012, Wolff *et al.*, 2013, Zhou *et al.*, 2014, Wolff *et al.*, 2014, Bui *et al.*, 2014, Xiong *et al.*, 2015, Lu *et al.*, 2016, Wolff, 2016) (Fig. 5.1); however, *in vivo* systems have proven to be more effective, with live rodent offspring (Zou *et al.*, 2009, Zhou *et al.*, 2014, Xiong *et al.*, 2015, Lu *et al.*, 2016), macaque monkey oocytes and embryos (Wolff *et al.*, 2013, Wolff *et al.*, 2014, Wolff, 2016) and immature human follicles (White *et al.*, 2012) being created. Two *in vivo* strategies have been described: (1) injection of species-matched OSCs into the ovaries of live animals with subsequent natural mating (Zou *et al.*, 2009, Zhou *et al.*, 2014, Xiong *et al.*, 2015, Lu *et al.*, 2016) or ovarian hyper-stimulation +/- IVF (White *et al.*, 2012, Wolff *et al.*, 2013, Wolff *et al.*, 2014, Wolff, 2016), and (2) injection of human OSCs into adult human ovarian cortex, with xenografting of the cortex into immunodeficient mice (White *et al.*, 2012).

In vitro culture systems have resulted in spontaneous and induced differentiation of OSCs into OLCs, which expressed markers of oocytes (Pacchiarotti *et al.*, 2010, Hu *et al.*, 2012, White *et al.*, 2012, Bui *et al.*, 2014), and of the creation of primordial follicle-like structures when OSCs and somatic cells were cultured together in a hanging drop (Pacchiarotti *et al.*, 2010) or on a membrane (White *et al.*, 2012). However, the ability of these *in vitro*-derived structures to be matured and fertilised has not been investigated and therefore these studies have not reached the "ultimate gold standard" that has been laid down for *in vitro*-derived germ cells: the production of live, healthy euploid offspring (Handel *et al.*, 2014).

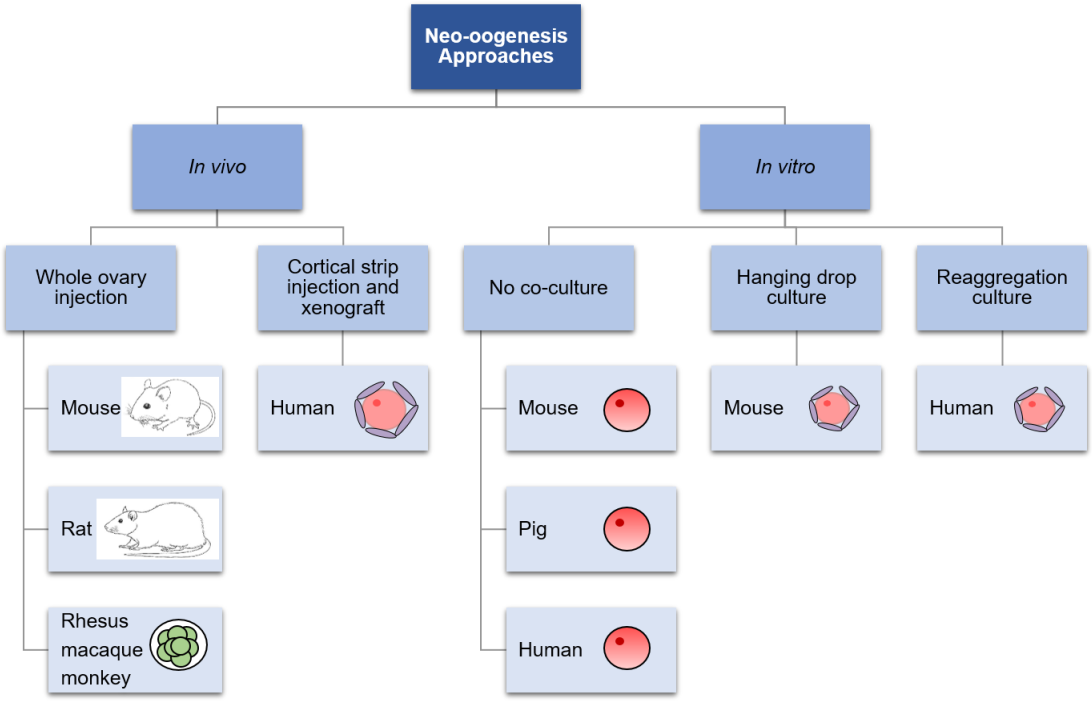


Figure 5.1. Approaches used to investigate the *neo*-oogenesis potential of OSCs. *In vivo* strategies have been more effective at demonstrating the functional capacity of OSCs to date, with live rodent offspring, macaque monkey embryos and immature human follicles formed. *In vitro* culture systems have resulted in OLCs and, when co-cultured with somatic cells, primordial follicle-like structures (Zou *et al.*, 2009, Pacchiarotti *et al.*, 2010, Hu *et al.*, 2012, White *et al.*, 2012, Bui *et al.*, 2014, Zhou *et al.*, 2014, Xiong *et al.*, 2015, Lu *et al.*, 2016, Wolff, 2016).

The development of an oocyte to maturity is a complex, multifaceted process under tight control by two-way communication with its surrounding somatic cells. One critical aspect is the precise timing of both meiotic arrest and subsequent resumption (Li and Albertini, 2013): if oocytes derived *in vitro* are to be considered "true" oocytes, then they must undergo meiosis correctly and demonstrate developmental competence (Handel *et al.*, 2014). A second critical feature concerns genomic imprinting and epigenetics (Anckaert *et al.*, 2013). For viable offspring to be produced, imprinting of

certain genes by epigenetic mechanisms such as DNA methylation has to occur in order that only the maternal or paternal allele is expressed: incorrect imprinting can have phenotypic consequences, such as Prader-Willi and Angelman syndromes (Anckaert *et al.*, 2013). It has been demonstrated that the initial steps of gametogenesis (i.e. PGC specification and maturation) can be induced convincingly *in vitro* with ESCs and iPSCs (Hayashi *et al.*, 2012), but to date *in vivo* conditions have been required for meiosis to occur and for the production of healthy offspring from *in vitro*-derived germ cells of any source (Handel *et al.*, 2014).

Nevertheless, an *in vitro* culture system would currently be the preferred approach if OSCs are to be used clinically: a xenograft system is not clinically acceptable and there would have to be rigorous testing of OSCs to ensure that autologous injection into ovaries would not result in a malignancy. Moreover, an *in vitro* culture strategy would also have the important basic science application of allowing investigation of the processes controlling oocyte development. The focus of this chapter is the *in vitro* assessment of putative bovine and human OSCs within two distinct, previously validated culture systems which support follicular development: (1) *in vitro* culture of ovarian cortical fragments, and (2) artificial "ovary" experiments utilising aggregation with somatic cells.

5.1.2 *In vitro* ovarian cortical culture

The ability to mature oocytes from the primordial stage to MII oocytes capable of fertilisation, completely *in vitro*, has both basic science and clinical import. It provides an experimental model for the enhancement of knowledge surrounding folliculogenesis and oocyte development and could also be beneficial in ovarian cortex cryopreservation fertility preservation strategies: for example, it may be useful for *in vitro* maturation of oocytes from ovarian tissue affected by malignancy that would not be safe for re-implantation. The complete process has only been performed in rodents to date (Eppig and O'Brien, 1996, O'Brien *et al.*, 2003). The replication of these results in species with a more prolonged folliculogenesis process has not been reported; however, a serum-free two-step culture system has been developed which successfully supports folliculogenesis and oocyte development from the primordial stage in both

cows (McLaughlin and Telfer, 2010) and humans (Telfer *et al.*, 2008) in a shorter timeframe than observed *in vivo*. This system involves the culture of fragments of ovarian cortex containing only primordial and transitory follicles, with excessive stroma removed to enhance follicle activation. After 6 days, preantral follicles are dissected from the fragments and cultured individually in culture medium supplemented with activin +/- FSH. These steps have facilitated the growth of bovine oocytes contained within primordial follicles to greater than 100µm in diameter in 15 days (McLaughlin and Telfer, 2010) and of human primordial follicles into antral follicles (Telfer *et al.*, 2008). In order to generate a MII oocyte, the next steps in this methodology would involve isolation and further *in vitro* growth of the cumulus-oocyte complex and subsequent *in vitro* maturation (IVM) of the oocyte, in preparation for fertilisation. It is evident that such a multi-step *in vitro* ovarian cortical culture system is required for provision of the changing requirements of a follicle during development.

This system could be used to assess the functional capacity of the putative bovine and human OSCs by injecting the cells into the cortical fragments and culturing them as above. This would require the ability to track the fate of the cells in order that they could be distinguished from native cells, including oocytes. Fluorescent labelling of the cells would enable this to occur. The incorporation of the cells into the established follicle culture system thus provides a theoretically straightforward methodology for the examination of *neo*-oogenesis.

5.1.3 Creating an artificial "ovary"

The creation of an artificial gonad by the aggregation of germ cells and somatic cells is well-established (O and Baker, 1978). Chimaeric re-aggregated rodent ovaries have been utilised to investigate several aspects of germ cell development by examining the interactions between germ cells and somatic cells of different sexes, different species and different strains of the same species (O and Baker, 1978, Eppig and Wigglesworth, 2000, Eppig *et al.*, 2002, Gittens and Kidder, 2005, Qing *et al.*, 2008). Such experiments involve the disaggregation of ovaries, separation of the germ cells and somatic cells and subsequent re-aggregation of the required cell populations by

centrifugation. The addition of phytohaemagglutinin, a lectin which agglutinates cells, has been found to aid the adhesion of the cells (Eppig and Wigglesworth, 2000). The artificial ovaries have been cultured on both agar blocks (O and Baker, 1978) and tissue culture membrane inserts (Eppig and Wigglesworth, 2000, Eppig *et al.*, 2002, Gittens and Kidder, 2005) overnight, before transplantation into ovariectomised mice. The approach supports follicular development and oocyte maturation, with live mouse offspring born after IVF (Eppig and Wigglesworth, 2000). A similar model has also been used entirely *in vitro*: genital ridge re-aggregates derived from embryonic mice supported the entry of germ cells into meiotic prophase I during a 4 – 5 day culture period on agar blocks, although follicular assembly was not reported (McLaren and Southee, 1997).

This culture system provides another opportunity to assess the ability of putative bovine and human OSCs to undergo *neo*-oogenesis. By substituting the germ cells for putative OSCs, the putative OSCs can be provided with a physiologically relevant ovary-like structure which should support folliculogenesis if the putative OSCs have germ cell function.

5.1.4 The role of somatic cells in *neo*-oogenesis

It is reasonable to hypothesise that the correct somatic cell environment is a fundamental requirement of *in vitro* approaches aimed at providing the right environment for OSCs to form oocytes. Not only are granulosa cells and theca cells required for the structural formation and endocrine function of follicles, but the surrounding stroma also has essential roles in structural support of, and bi-directional paracrine communication with, the follicle (Tagler *et al.*, 2011). All of these types of somatic cells thus contribute to the healthy growth and development of a follicle *in vivo* (Hummitzsch *et al.*, 2015). Research into the oogenesis potential of mouse ESCs has demonstrated the importance of somatic cells in the induction of ESCs to form OLCs. Co-culture of ESCs with granulosa cells from newborn mice stimulated the expression of oocyte-specific markers (e.g. *GDF-9* and *ZP1-3*) (Qing *et al.*, 2007). This upregulation was not detected if ESCs were cultured in granulosa cell-conditioned medium alone, providing evidence that cell-to-cell contact between ESCs

and somatic cells is essential for differentiation. Furthermore, as discussed in section 1.2.6, aggregation of mouse ESC- and iPSC-derived PGCLCs with developmentally-appropriate fetal gonadal somatic cells supported the formation of developmentally competent oocytes which were fertilised to produce healthy pups (Hayashi *et al.*, 2012). Thus, it is extremely unlikely that OSCs would differentiate into healthy, developmentally competent oocytes without the presence of supportive somatic cells.

5.1.5 Aims of this chapter

Although OLCs, as classified by morphology, were spontaneously formed during *in vitro* culture of putative bovine and human OSCs, characterisation of the cells did not demonstrate expression of the definitive oocyte marker, *ZP3*. As such, the ability of the cells to undergo *neo*-oogenesis was not proven and, thus, the overall aim of the experiments in this chapter was to assess this important aspect of the research. We opted to use an exclusively *in vitro* approach to investigate the potential of the cells as, if the cells are to be used clinically, this is a more ethically acceptable strategy than current *in vivo* techniques and is thus more clinically relevant. Due to the expertise of Prof. Telfer's group in *in vitro* bovine and human follicle culture (Telfer *et al.*, 2008, McLaughlin and Telfer, 2010), the initial aim was to incorporate the putative OSCs into this system. However, a differing approach was subsequently required and a well-established re-aggregation technique was modified for *in vitro* use, with the aim of providing an improved somatic cell and extracellular environment for the putative OSCs.

5.2 Materials and Methods

Two different *in vitro* culture approaches were used to assess the capacity of putative bovine and human OSCs to undergo oogenesis: (1) injection of putative OSCs into adult ovarian cortex and (2) the creation of artificial ovaries using putative OSCs and fetal somatic cells.

5.2.1 Injection experiments

The first approach involved the injection of putative OSCs into adult ovarian cortical fragments and subsequent *in vitro* culture as previously described (Telfer *et al.*, 2008, McLaughlin and Telfer, 2010; Fig. 5.2). Fluorescently-labelled putative OSCs were utilised to allow the cells to be tracked within the tissue. Injection experiments were performed with Dr. Marie McLaughlin (from Prof. Telfer's group).

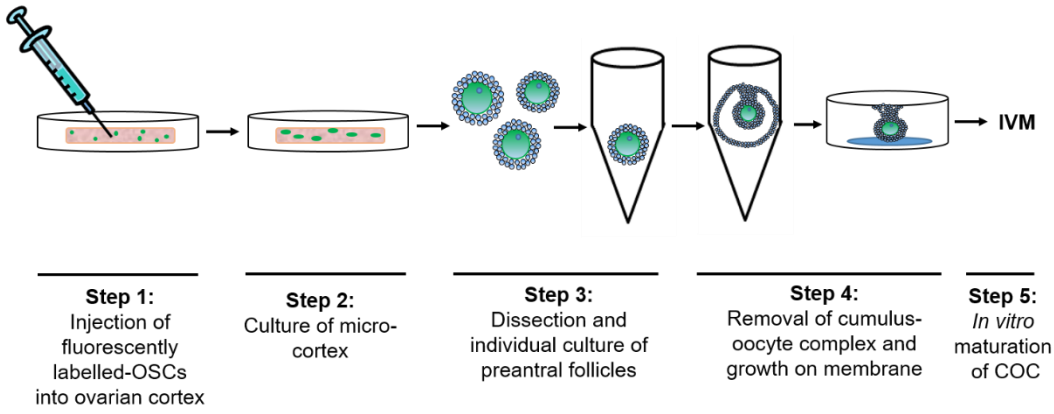


Figure 5.2. Schematic of the injection experiments intended to assess the ability of putative OSCs to undergo *neo*-oogenesis. The aim was to inject OSCs transduced to express fluorescent markers into ovarian cortex fragments and culture the fragments *in vitro* for 1-2 weeks. Developing preantral follicles would be dissected from the fragments and cultured individually, before the cumulus-oocyte complex (COC) was released and cultured on a membrane. The final step would be *in vitro* maturation (IVM) of the oocyte. Ultimately, Steps 4 and 5 were not performed.

5.2.1.1 Bovine experiments

Cultured bovine putative OSCs (Cell Line 3, Population B, P10 and P15) were transduced with GFP-containing lentivirus and purified so that only GFP-positive cells were used in the experiments (see section 2.5.1). Due to the detrimental effect of FACS on cell health, GFP-expressing cells were plated down after purification to ensure that only live cells were used in the experiments. One week later, vitrified adult bovine ovarian cortex was thawed as per section 2.2 and the pieces were examined under the light microscope as previously described (Telfer *et al.*, 2008, McLaughlin and Telfer, 2010), with excision of visible growing follicles. The GFP-expressing cells were trypsinised, centrifuged at 800 x g for 5 mins and re-suspended in 1x PBS. The cells were counted by haemocytometer (as per section 2.5.1) and a small amount of trypan blue solution (Sigma-Aldrich) was added so that the cell suspension could be visualised under a dissecting microscope during injections. The cell suspension was drawn up into a Nanofil 10µl syringe (World Precision Instruments Ltd.) using a 26G Nanofil needle (World Precision Instruments Ltd.). The needle was then replaced with a 35G bevelled Nanofil needle (World Precision Instruments Ltd.) and cells were injected into the thawed tissue pieces (n = 97). Cells were either injected directly into small fragments of cortex (10mm x 1mm x 1mm) or into larger pieces of cortex (10mm x 10mm x 1mm) which were then cut with a sterile scalpel into smaller fragments. The amount of cell suspension injected into each fragment was recorded so that the number of cells injected could be calculated. One to nine microlitres were injected per small fragment (mean: 5µl) and 11 – 14 µl (mean: 12µl) were injected per large fragment (which were each then cut into 10 smaller fragments). The negative control comprised a vehicle control group where tissue fragments were injected with trypan blue-dyed PBS only (n = 32) and a non-injected group where tissue fragments were cultured without any injections (n = 2). The tissue fragments were cultured in 24 well plates in 300µl of culture medium (comprising McCoy's 5A (modified) medium (containing HEPES; Life Technologies) supplemented with 1mg/ml BSA, 100µg/ml penicillin G, 100µg/ml streptomycin sulphate, 3mM L-glutamine, 2.5µg/ml transferrin, 4ng/ml selenium, 10ng/ml insulin, 50µg/ml ascorbic acid and 50ng/ml recombinant human FSH (rhFSH; all Sigma-Aldrich) for 7 or 14 days, with half of the

culture medium replaced with fresh medium on alternate days. Fragments were analysed for the presence of preantral follicles after 7 days: observed follicles were excised using 25G needles (BD Biosciences) attached to 1ml syringe barrels and cultured separately for a further 7 days. Fragments and excised follicles were fixed for 24 hours in 4% NBF and subsequently processed for immunohistochemical analysis.

5.2.1.2 Human experiments

Lentiviruses containing mCherry were used to transduce cultured human putative OSCs (Patient 1, Population B, P9) and mCherry-expressing cells were purified as per section 2.5.1. Cells were subsequently trypsinised, re-suspended in 1x PBS, counted using a haemocytometer and the cell suspension dyed with trypan blue as per section 5.2.1.1. Vitrified adult human ovarian cortex from a 33 year old women undergoing elective Caesarean section was thawed as per section 2.2 and cut into 10mm x 1mm x 1mm fragments. Injections were performed as per section 5.2.1.1, with two groups generated: putative OSC-injected tissue (n = 6) and vehicle control-injected tissue (n = 4). Ten to fifteen microlitres (mean: 13µl) were injected into each fragment. The tissue fragments were cultured in the culture medium described in section 5.2.1.1 for 7 days, with half of the medium replaced with fresh medium on alternate days. The fragments were then fixed for 24 hours in 4% NBF before being processed for IHC.

5.2.1.3 Immunohistochemistry

Tissue fragments were manually embedded, sectioned and mounted as per section 2.6.1. DAB IHC was performed to identify GFP or mCherry-expressing cells using avidin/biotin peroxidase detection (see section 2.6.4.1). To detect GFP, either a rabbit polyclonal antibody against GFP (#2555, Cell Signaling Technology; used at 1:800) or a mouse monoclonal against GFP (MAB3580, Merck Millipore; used at 1:500) was utilised. A rabbit polyclonal against RFP (600-401-379, Rockland; used at 1:100) was used to detect mCherry. A goat anti-rabbit secondary antibody and normal goat serum or a horse anti-mouse secondary and normal horse serum (all Vectastain® ABC Kit) was used as appropriate. The primary antibody was omitted for the purposes of a negative control. The protocol was optimised, with many of the steps subsequently

being adjusted, including an increase in the length of time for serum blocking and a reduction in DAB incubation time. The final attempt at achieving adequate analysis involved the addition of a further avidin/biotin blocking step prior to the primary antibody incubation step, with slides incubated at room temperature for 15 mins with avidin solution (Vector Laboratories), washed twice with TBST and incubated for a further 15 mins at room temperature with biotin solution (Vector Laboratories).

5.2.2 Artificial ovary experiments

The functional capacity of cultured putative OSCs from both species was subsequently investigated by aggregating fetal somatic cells and putative OSCs to create an artificial "ovary", using a modified version of a previously published rodent ovary re-aggregation protocol (Eppig and Wigglesworth, 2000, Eppig *et al.*, 2002). To ensure the culture model was effective, experiments were initially performed in mouse, using mouse somatic cells and mouse germ cells (rather than OSCs; Fig. 5.3).

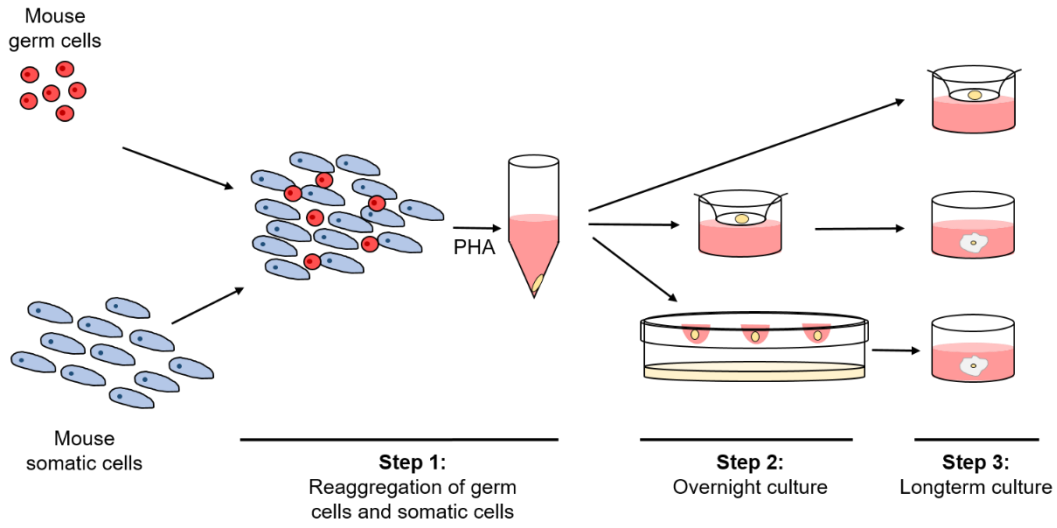


Figure 5.3. Schematic representing the mouse artificial "ovary" experiment. Mouse germ cells and somatic cells were separated by dissociation of neonatal mouse ovaries and re-aggregated in the presence of phytohaemagglutinin (PHA). The pellets were then cultured entirely on a membrane, or placed on a membrane or in a hanging drop for overnight culture, before longer term culture in collagen.

5.2.2.1 Murine experiments

Mouse ovaries were obtained from post-natal CD1 mice (post-natal days 1 – 5; Charles River). The mice were housed in the animal house at Chancellor's Building, University of Edinburgh. They were fed *ad libitum* and experienced a 12 hour light-dark cycle at 19-21ºC, in accordance with UK Home Office and University of Edinburgh ethical standards. Mice were culled by cervical dislocation prior to transportation to the laboratory. Female mice were identified by examination of the anogenital distance. Ovaries were then dissected out under a Leica MZ12.5 stereo-microscope (Leica) in a laminar flow hood using sterile forceps.

A previously published disaggregation and re-aggregation protocol (Eppig and Wigglesworth, 2000, Eppig *et al.*, 2002) was used with some modifications. All steps were performed under sterile conditions in a laminar flow hood. Ovaries were placed in DPBS containing 1mg/ml BSA (DPBS/BSA) and extraneous tissue, including the ovarian bursae, was dissected from the ovary using sterile 25G needles attached to 1ml syringe barrels (BD Biosciences). The ovaries were washed in DPBS/BSA and then dissociated in 0.05% trypsin-EDTA containing DNase I (1:100 DNase I:trypsin ratio) by incubating the ovaries at 37ºC / 5% CO_2 for 15 mins. The ovaries were then pipetted repeatedly for several minutes before being incubated for a further 5 mins. This step was repeated once more before the cell suspension was filtered through a 100µm filter (Partec) into a 15ml centrifuge tube and the trypsin solution was neutralised with an equal volume of holding medium (M199 medium supplemented with 10% (v/v) FBS, 1x penicillin-streptomycin (Life Technologies) and 0.22% (v/v) sodium DL-lactate syrup (60% (w/w) stock; Sigma-Aldrich)). The suspension was centrifuged at 2000rpm for 5 mins, the cell pellet was re-suspended in fresh holding medium and the cell suspension was then placed in a 35mm culture dish (Corning). The dish was incubated overnight at 37ºC / 5% CO_2 and the following day non-adherent cells (which include germ cells, blood cells and dead cells) were removed and placed in a separate culture dish with fresh holding medium. The adherent monolayer of cells (i.e. somatic cells) were washed twice with DPBS/BSA and trypsinised. After neutralisation with holding medium, the cell were centrifuged at 2000rpm for 5 mins and the pellet was re-suspended in fresh holding medium. Both non-adherent and somatic cells were then

incubated at 37ºC / 5% CO_2 for a further 5-6 hours. To ensure complete separation of germ cells and somatic cells, the somatic cells were washed twice more and trypsinised again. The cell suspension was neutralised with holding medium and following centrifugation at the above conditions and resuspension in fresh holding medium, the cells were counted using a haemocytometer (see section 2.5.1). The germ cells were also centrifuged, re-suspended in holding medium and counted (distinguished from other cells by their morphology) and the desired ratio of somatic cells to germ cells was placed in a 0.5ml microfuge tube (Eppendorf). Due to lack of information in the adapted protocols (Eppig and Wigglesworth, 2000, Eppig *et al*. 2002) regarding optimal ratios, a ratio of 10:1 somatic cells to germ cells was arbitrarily chosen to be used due to cell availability. If, however, germ cell numbers were low, then ratios up to 50:1 were used. Pellets contained between 55000 – 226000 cells in total. PHA (Sigma-Aldrich) was added at final concentration of 35µg/ml to promote re-aggregation of the cell populations. The microfuge tube was centrifuged at 10000rpm for 30 secs, rotated 180º and re-centrifuged at the same conditions to aid compaction, and thus handling, of the pellet (termed positive pellet in this Chapter). Somatic cell-only pellets were also generated as negative controls. The pellet was removed with a pipette and to encourage the pellet to develop a three-dimensional (3D) structure it was placed on either a membrane (initial experiments, as per Eppig & Wigglesworth (Eppig and Wigglesworth, 2000)) or in a hanging drop of culture medium (latter experiments) overnight.

For membrane experiments, pellets were placed on Transwell®-COL collagen-coated 3.0µm pore membrane inserts (Corning) which had been pre-equilibrated with culture medium (phenol red-containing Waymouth's medium (Life Technologies) supplemented with 10% (v/v) FBS and 1 x penicillin-streptomycin) in a 12 well plate. Culture medium (1.5ml) was placed below the membrane and approximately 50µl of culture medium was placed on top of the pellet. For hanging drop experiments, pellets were aspirated from the tube in 30µl of culture medium and placed in the drop of medium onto the inside of the lid of a 100mm culture dish. The lid was then inverted onto the dish which was filled with sterile DPBS to maintain hydration of the tissue. Both culture plates and dishes were incubated overnight at 37ºC / 5% CO_2.

The following day, the pellets were encapsulated in 3mg/ml collagen I (rat tail collagen I, Thermo Scientific) to confer an extracellular structural support. The pellet was aspirated from the membrane or hanging drop in minimal medium and placing into a droplet of collagen. The collagen encapsulated pellet was then incubated at 37ºC / 5% CO_2 for 10 mins to allow the collagen to solidify before being transferred to fresh culture medium in a 24 well plate. In one experiment, the pellets were kept on the membranes for the entire culture period, without collagen encapsulation. Pellets were cultured for 7-14 days with 100µl fresh medium added on alternate days, before being fixed in either 4% NBF overnight (initial experiments) or Bouin's for 3 hours (latter experiments).

The number of replicates for each culture procedure is detailed in Table 5.1.

Table 5.1. Number of replicates for each *in vitro* re-aggregation culture protocol.

Hanging Drop or Membrane Overnight	Collagen or Membrane for Rest of Culture	Length of Culture (days)	No. of Positive Pellets	No. of Negative Controls
Membrane	Membrane	7	3	1
Membrane	Collagen	7	8	4
Hanging Drop	Collagen	10	2	1
		14	4	4

Imaging of the pellets was performed during and at the end of experiments using an inverted microscope (Axiovert 200) and an attached Hamamatsu camera. Scale bars were applied using the Zen software.

5.2.2.2 Bovine and human experiments

For bovine and human experiments, fetal somatic cells had first to be isolated, before the aggregation experiments could be performed (Fig. 5.4).

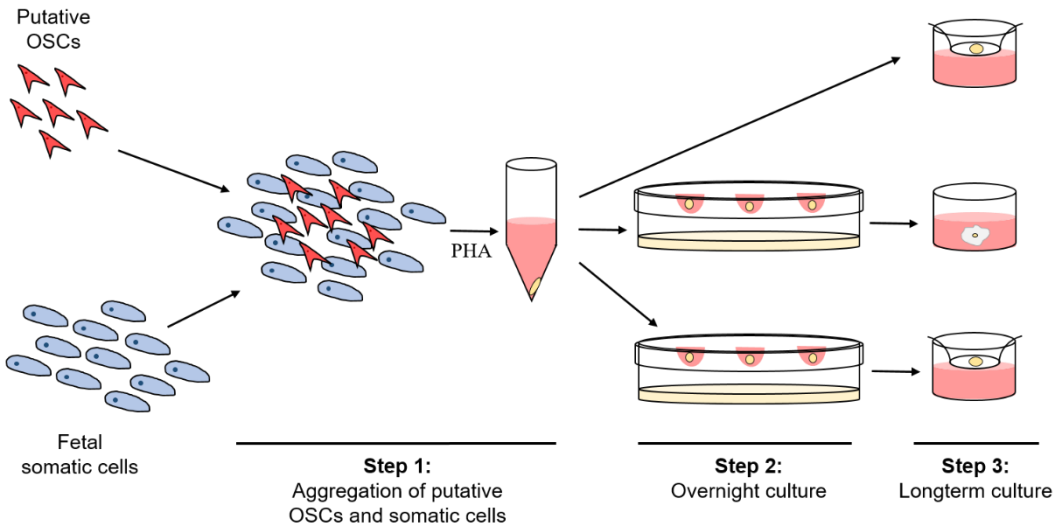


Figure 5.4. Schematic representing the bovine and human artificial "ovary" experiments. Putative OSCs and fetal somatic cells were re-aggregated in the presence of PHA. The pellets were then cultured entirely on a membrane, or placed in a hanging drop for overnight culture, before longer term culture in collagen or on a membrane.

5.2.2.2.1 Fetal somatic cell isolation and culture

Bovine fetal ovarian somatic cells were derived from the ovaries of a 164 day old fetus (retrieved as per section 2.1.1). Derivation was performed by Kelsey Grieve (PhD student in Prof. Anderson's group). The ovaries were placed on separate dimple slides and disaggregated enzymatically in 200µl of 10mg/ml collagenase (Type IV; Sigma-Aldrich) in HBSS (minus Ca^{2+} and Mg^{2+}) and mechanically with 19G needles (BD Biosciences). The cell suspension was placed in a 1.5ml microfuge tube and incubated at 37ºC at 900rpm for 10 mins in a Thermomixer® (Eppendorf), with frequent pipetting to aid disaggregation. Two hundred microlitres of 7mg/ml stock of DNase I was then added and a further 5 min incubation performed in identical conditions. The cell suspension was centrifuged at 500 x g for 5 mins, the supernatant discarded and the pellet re-suspended in 1ml of HBSS. This step was repeated once more before a

further centrifugation at 500 x g for 5 mins was performed. The pellet was then washed in 1ml of somatic cell culture medium, comprising DMEM (phenol-red free, high glucose) supplemented with 10% (v/v) FBS, 2mM (v/v) L-glutamine, 1x NEAA and 1x penicillin/streptomycin/amphotericin (all Life Technologies). The suspension was filtered using a 70µm cell strainer (BD Biosciences) and centrifuged again at 500 x g for 5 mins. The cell pellet was then re-suspended in somatic cell culture medium and placed in 8 wells of a 12 well culture plate (Corning). The cells were incubated at 37ºC / 5% CO_2 overnight. The following day, the cells were washed with fresh culture medium twice to remove dead and non-adherent cells. Fresh culture medium was then added and the plate was placed back in the incubator. Subsequently, the cells were passaged using 0.25% (v/v) trypsin-EDTA or cryopreserved as required. For cryopreservation, cells were trypsinised, centrifuged at 800 x g for 5 mins and the pellet re-suspended in 1ml Bambanker^{TM} cell freezing medium (Lymphotec Inc.). The cell suspension was placed in a cryovial and stored at -80ºC.

Human fetal ovarian somatic cells were derived from the ovaries of three morphologically normal fetuses by Dr. Rosey Bayne (from Prof. Anderson's group; see section 2.1.2 for further details). The above methodology was utilised with the following modifications: due to the amount of tissue, only 50µl collagenase and DNase I were used per ovary and cells were placed in only 2 wells of a 12 well plate. Passaging and cryopreservation was performed as above.

5.2.2.2.2 Bovine aggregation experiments

Two bovine aggregation experiments were performed using fetal bovine somatic cells (P3) and rhodamine-expressing cultured putative bovine OSCs (Cell Line 4, P16 and P19). Cultured putative OSCs were labelled with rhodamine as per section 2.5.2 and examined under an inverted microscope (Axiovert 200) to ensure fluorescence was detectable in the majority of cells prior to use in the experiments. In the first experiment, the cells were aggregated as per section 5.2.2.1, cultured overnight in a hanging drop and in collagen for a further 7 days. For the second experiment, the cells were aggregated as above, but in a 5:1 somatic cell to germ cell ratio and in OSC

culture medium (see section 2.4). The pellets contained 73000 – 85000 cells. The pellet was cultured on a membrane for 7 days with no encapsulation in collagen. Bouin's was used as a fixative in both experiments. The number of replicates for each experiment is shown in Table 5.2.

Imaging of the pellets was performed using an inverted microscope (Axiovert 200) and an attached Hamamatsu camera. Scale bars were applied using Zen software.

5.2.2.2.3 Human aggregation experiments

Four human aggregation experiments were performed using fetal human somatic cells (three different cell lines: Cell Line 1 from 16^{+1} week fetus, Cell Line 2 from 17^{+3} week fetus and Cell Line 3 from 17^{+5} week fetus) and cultured putative human OSCs. The aggregations were performed as per section 5.2.2.1 and the details of each experiment are tabulated below (Table 5.3). The pellets were comprised of between 58,000 and 550,000 cells.

Imaging of the pellets was performed during and at the end of experiments using a Leica MZ FLIII stereo-microscope (Leica), the PM Capture Pro 6.0 software (Photometrics) and an attached CoolSNAP camera (Photometrics).

5.2.2.2.4 Chimaeric aggregation experiment

One chimaeric aggregation experiment was performed to assess whether fetal bovine somatic cells could support folliculogenesis. Fetal bovine somatic cells (P3) were aggregated with mouse germ cells (derived as per section 5.2.2.1) at a 30:1 ratio. The pellet was cultured in a hanging drop overnight before encapsulation in collagen and cultured for 14 days in Waymouth's culture medium.

Table 5.2. Experimental details of the two bovine aggregation experiments.

Expt. No.	Somatic Cell Line Passage	Putative OSC Cell Line (passage)	Ratio of Somatic Cells to OSCs	Hanging Drop or Membrane Overnight	Collagen or Membrane for Rest of Culture	Culture Medium	Length of Culture	No. of Positive Pellets	No. of Negative Controls
1	3	4 (P16)	10:1	Hanging Drop	Collagen	Waymouth's	7 days	2	2
2	3	4 (P19)	5:1	Hanging Drop	Membrane	OSC culture medium	7 days	2	2

Table 5.3. Experimental details of the four human aggregation experiments.

Expt. No.		Somatic Cell Line (passage)	Putative OSC Cell Line (passage)	Ratio of Somatic Cells to OSCs	Hanging Drop or Membrane Overnight	Collagen or Membrane for Rest of Culture	Culture Medium	Length of Culture	Fixative	No. of Positive Pellets	No. of Negative Controls
1	**a**	1 (P14)	Patient 1 (P8)	5:1	Membrane	Membrane	OSC culture medium	7 days	4% NBF	1	1
	b	2 (P10)								1	1
2		3 (P5)	Patient 8 (Pop B; P4)	10:1	Hanging Drop	Collagen	Waymouth's	7 days	Bouin's	1	0
								14 days		1	1
3		1 (P7)	Patient 1 (P8)	10:1	Hanging Drop	Membrane	OSC culture medium	7 days	Bouin's	2	2
4		1 (P9)	Patient 1 (P9)	10:1	Hanging Drop	Membrane	OSC culture medium	7 days	Bouin's	2	2

5.2.2.3 Immunohistochemistry

Due to their small size, pellets were embedded in 2.5% (w/v) agarose in 1x PBS prior to processing into paraffin wax blocks to ease transfer into blocks. Embedded pellets were placed in 70% ethanol and mechanically processed, sectioned and mounted as per section 2.6.1. Serial sectioning was performed with every 5th section placed on a separate slide for haematoxylin and eosin (H&E) staining (performed as per section 2.6.3).

Pellets from human aggregation experiment 1(a) were analysed by DAB detection for the presence of LIN28- and DAZL-expressing cells using avidin/biotin peroxidase detection as per section 2.6.4.1. A rabbit polyclonal antibody against LIN28 (ab46020, Abcam; used at 1:500) and a mouse monoclonal against DAZL (MCA2336, AbD Serotec; used at 1:200) were used, with the appropriate goat anti-rabbit or horse anti-mouse secondary antibody (Vectastain® ABC Kit).

5.2.2.4 Immunofluorescence

One mouse pellet (cultured in a hanging drop, then in collagen) and one human pellet (Expt 1(a)) was analysed for DDX4 expression using IF as per section 2.6.5. Normal chicken serum, a rabbit polyclonal antibody against DDX4 (ab13840, Abcam; used at 1:200), a chicken anti-rabbit peroxidase secondary antibody (sc-2963, Santa Cruz; used at 1:200), a red tyramide signal identification kit and DAPI nuclear counterstain were utilised. The primary antibody was omitted for a negative control.

One bovine pellet was also analysed for rhodamine expression using a simplified IF protocol. Slides were dewaxed and rehydrated as per section 2.6.2, before a DAPI nuclear counterstain (1:1000, diluted in PBS) was applied for 10 mins at room temperature. After two washes in PBS, slides were mounted with PermaFluor^{TM} and stored at 4°C in the dark.

5.2.3 Histological Analysis

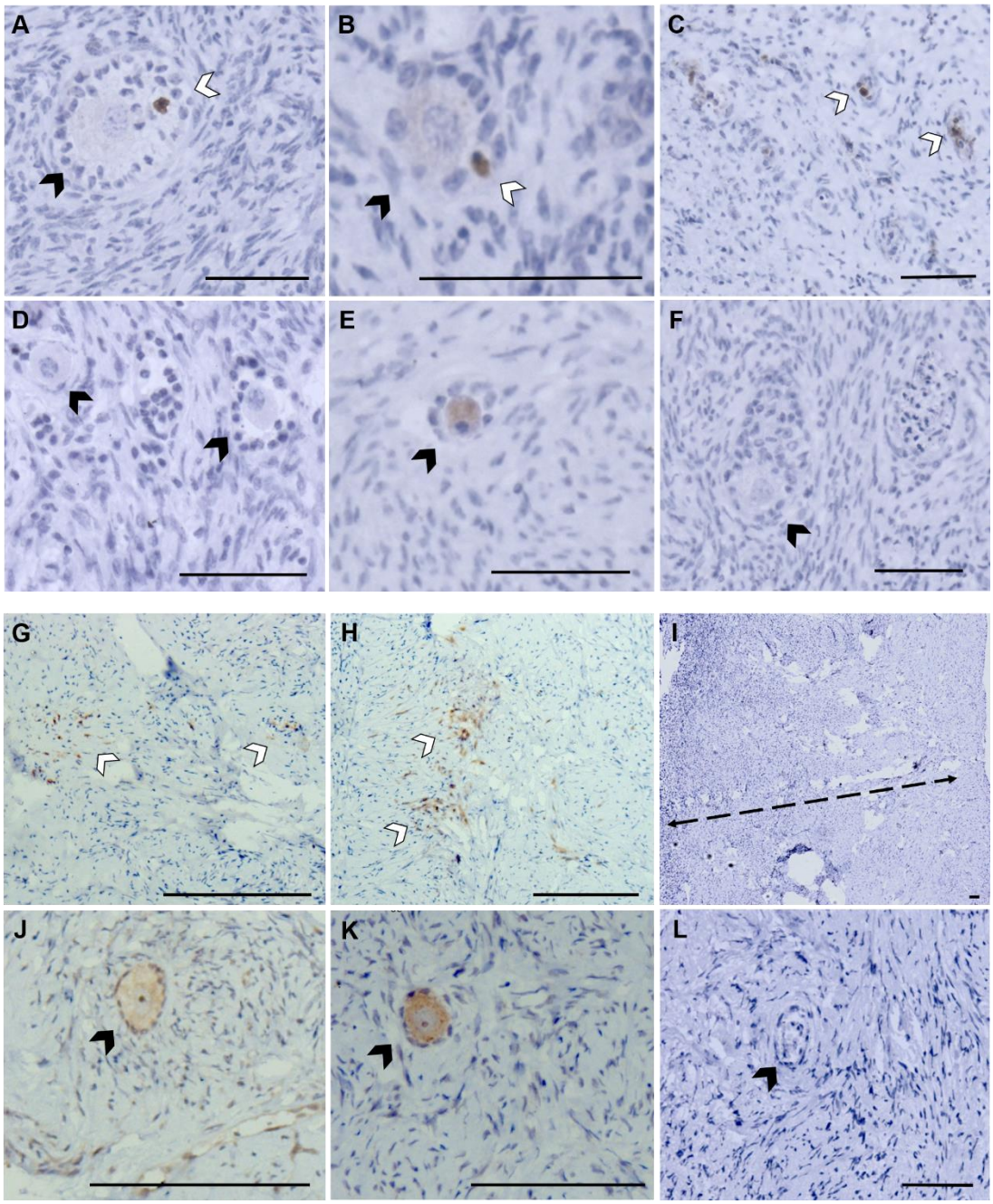
IHC analysis was performed using a light microscope as per section 2.8.1 and IF analysis was performed using a confocal laser scanning microscope as per section 2.8.2.

5.3 Results

5.3.1 Injection experiments

Between 500 and 600 putative OSCs were injected into each tissue fragment in both bovine and human experiments; however, it was a technically difficult procedure so this may be an overestimate as some of the cell suspension unintentionally extruded into the surrounding medium instead of into the tissue. This remained the case even if cells were injected into larger pieces of cortex, which were then cut into smaller fragments. In the GFP-labelled experiments, there was an infrequent occurrence of DAB-positive cells closely associated with somatic cells and adjacent to native follicles (Fig. 5.5). In mCherry-labelled experiments, collections of DAB-positive cells were observed around the sites of injection, with no association with any other cells or structures (Fig. 5.5). However, it was not possible to analyse the tissue by IHC reliably as in both GFP and mCherry experiments, at least one oocyte per section of tissue was observed to be DAB-positive in vehicle-injected tissue and non-injected tissue (Fig. 5.5). When the primary antibody was omitted, no DAB staining was observed, indicating that the cause of the oocyte staining in vehicle-injected tissue was non-specific binding of the primary antibody. This non-specific antibody binding occurred despite numerous methodological alterations, including increased blocking steps. Moreover, the structural integrity of the human tissue was poor, likely due to the necessary handling of the tissue during injections (Fig. 5.5). These findings, coupled with the observation that transduction appeared detrimental to the health of the putative OSCs (see Chapter 3), resulted in a different functional testing approach being undertaken.

Figure 5.5. Immunohistochemical analysis of bovine (A – F) and human (G – L) injection experiments using an antibody against GFP and mCherry respectively. **(A, B)** Small DAB-positive cells (white arrows) in association with somatic cells and adjoining native follicles (black arrows) were observed rarely. **(C)** Other DAB-positive cells were detected throughout the cortex with no obvious association with other structures. **(D, E)** Vehicle-injected tissue acted as a negative control; however, non-specific binding to some oocytes was observed despite methodological alterations. Black arrows denote native immature bovine follicles. **(F)** The primary antibody was omitted as an additional negative control. No DAB staining was observed in oocytes (black arrow), indicating that the cause of the DAB staining in vehicle-injected tissue was non-specific binding of the primary antibody. **(G, H)** Collections of DAB-positive cells (white arrows) were detected around the injection sites, which demonstrated no association with any other structures. **(I)** Manipulation of the tissue was detrimental to the structural integrity of the cortex. A track mark from the insertion of the needle is denoted by the black arrow. **(J)** Vehicle-injected and **(K)** non-injected tissue was used as negative controls; however, non-specific binding to oocytes was again observed despite methodological alterations. Black arrows denote native immature human follicles. **(L)** The primary antibody was omitted as an additional negative control. Again, no DAB staining was detected (black arrow = atretic follicle). Scale bars = 50µm.


A
B
C
D
E
F
G
H
I
J
K
L

5.3.2 Artificial ovary experiments

5.3.2.1 Murine experiments

Murine re-aggregation experiments were utilised to ensure that the *in vitro* artificial ovary culture system could support *neo*-folliculogenesis prior to utilising bovine and human cells. Different techniques were utilised in an attempt to optimise the system. Cell pellets were flat and disc-shaped immediately after re-aggregation, therefore in order to form a more physiological relevant spherical pellet, overnight culture on either membranes or in hanging drops was performed, with the latter proving the more straightforward and effective method (Fig. 5.6). When pellets were cultured on a membrane for the entire culture period, the pellets grew out over the membrane and did not maintain a coherent structure, therefore histological analysis of these pellets was not possible.

When pellets were encapsulated in collagen, they kept their spherical structure. Moreover, the pellets used the surrounding collagen as an extracellular matrix (ECM), with protrusions of cells observed growing into the collagen (Fig. 5.6). Growing follicles could be seen macroscopically (Fig. 5.7(a)) and when the fixative was changed to Bouin's, morphologically healthy follicles were observed, in primordial, transitory and primary follicle stages (Fig. 5.7(b)). Germ cells were located peripherally within the pellet whilst the centre of the pellet appeared less healthy on H&E staining, with pyknotic nuclei observed. Furthermore, although healthy follicles were formed with maintenance of DDX4 expression (Fig. 5.8), there were evidently high germ cells losses in the culture system: only 0.3% ± 0.2% (mean ± S.E.M.) of germ cells included in the initial pellets were observed on histological analysis at the end of the culture period.

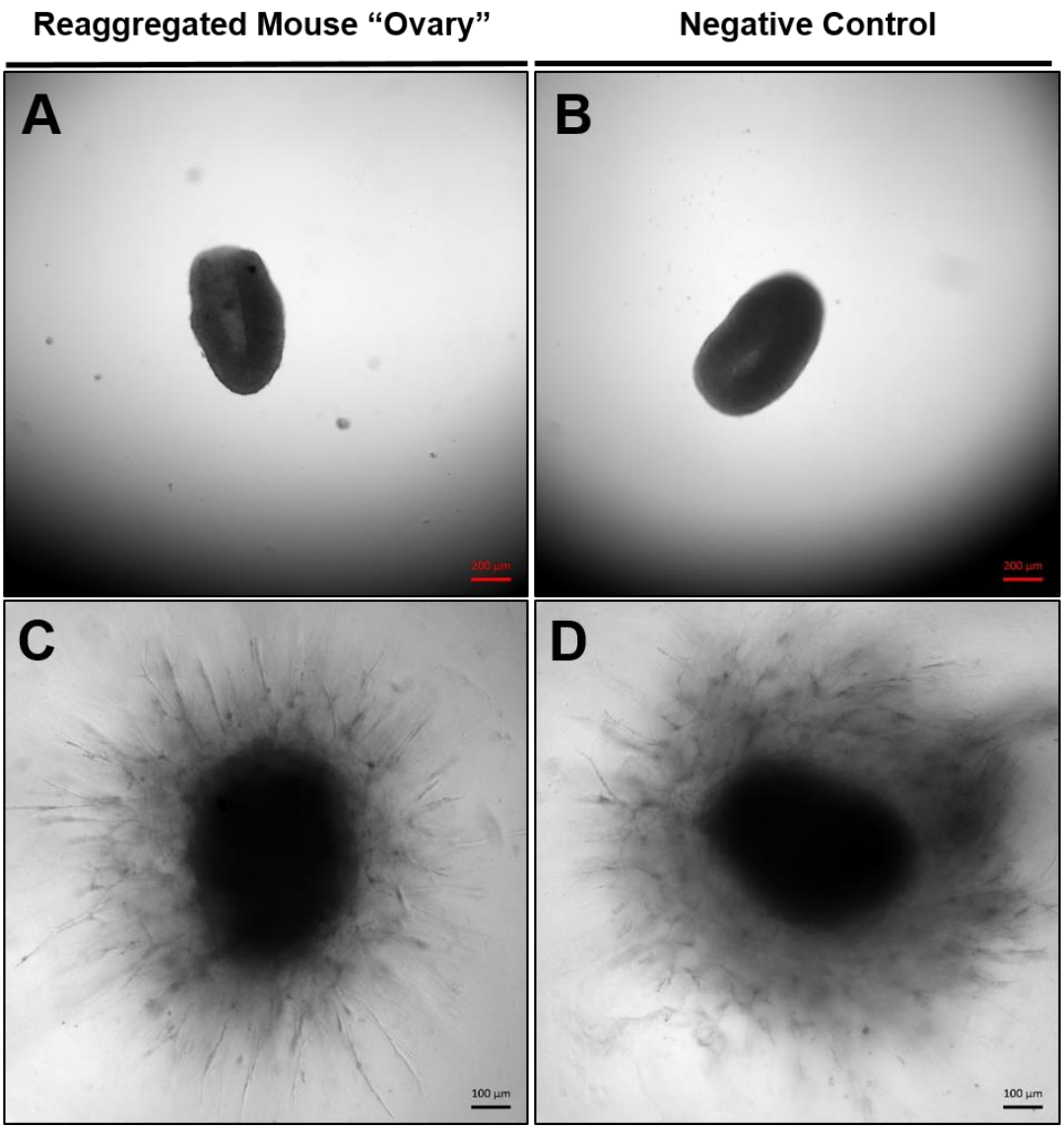
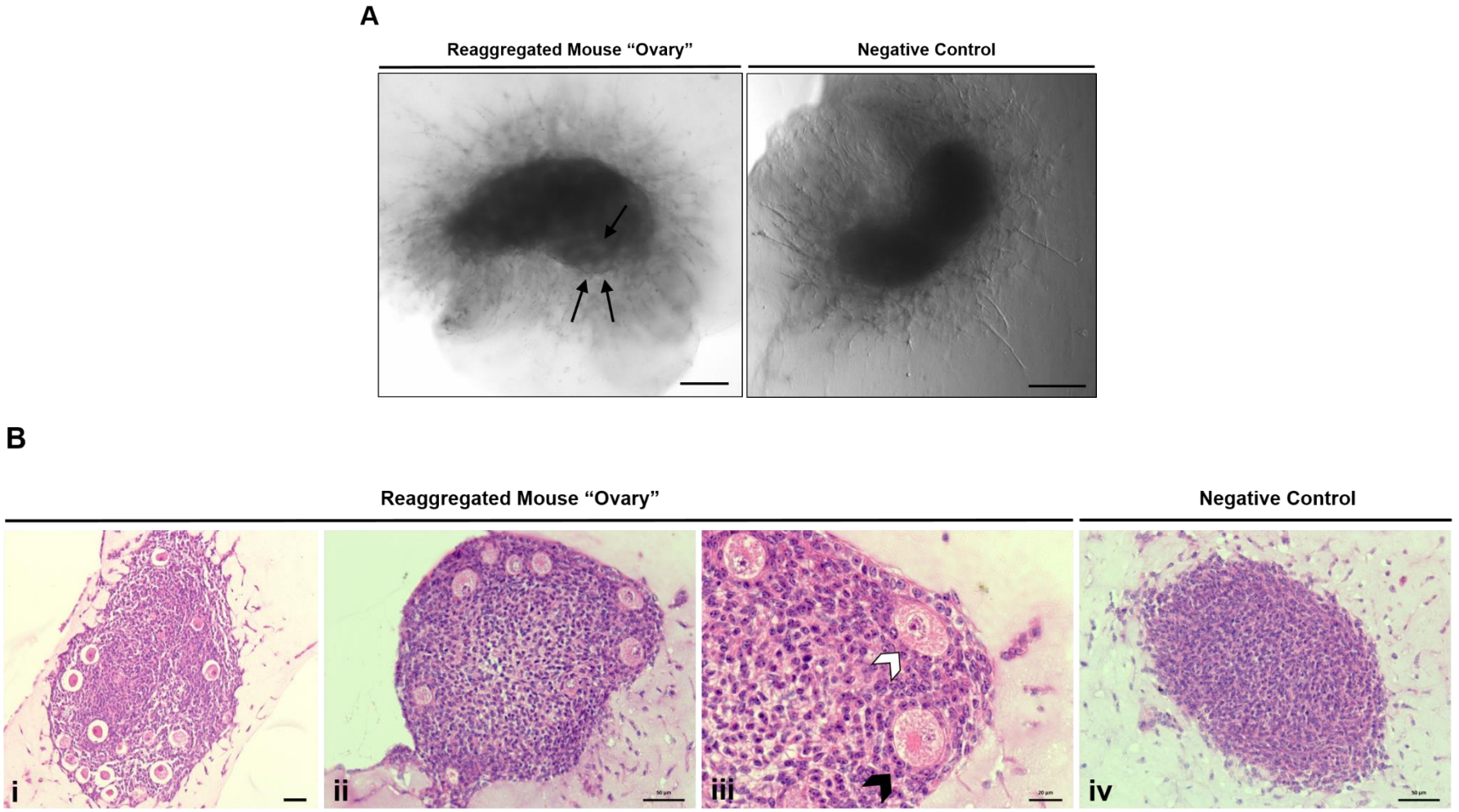


Figure 5.6. Bright field images of re-aggregated mouse pellets and negative controls (i.e. somatic cells only). (A, B) After 24 hours in hanging drop culture, the flat pellets became spherical in structure. **(C, D)** Subsequent culture in collagen droplets resulted in the somatic cells of the pellets projecting into the collagen. Scale bars = 100µm.

Figure 5.7. The *in vitro* culture system supported follicular development. (A) Bright field images of a re-aggregated pellet and corresponding negative control, demonstrating translucent areas within the positive pellet indicative of follicles (black arrows). Scale bars = 200µm. **(B)** H&E analysis of re-aggregated mouse pellets. **(i)** 4% NBF was found to be a poor fixative, with oocytes observed to be in poor association with surrounding somatic cells. Scale bar = 50µm. **(ii)** Bouin's fixative demonstrated morphologically healthy immature murine follicles, located peripherally within the pellet. The centre of the pellet was less dense and contained cells with pyknotic nuclei. Scale bar = 50µm. **(iii)** An enlarged area of the section in **(ii)** demonstrating a healthy primary follicle (black arrow) and transitory follicle (white arrow). Scale bar = 20µm. **(iv)** A negative control pellet demonstrating the presence of somatic cells only. Scale bar = 50µm.


A
Reaggregated Mouse "Ovary"
Negative Control
B
Reaggregated Mouse "Ovary"
Negative Control
i
ii
iii
iv

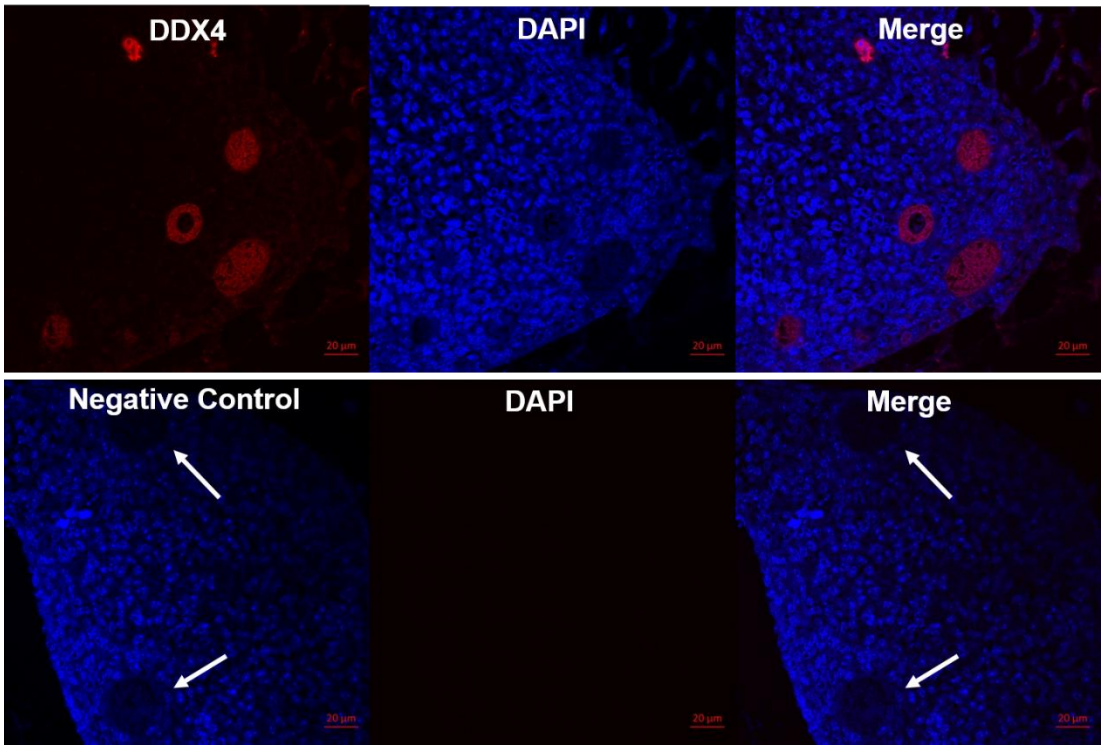


Figure 5.8. Immunofluorescence images demonstrating that mouse oocytes expressed DDX4 (red) within re-aggregated positive pellets. DAPI (blue) was used as a nuclear counterstain. The primary antibody was omitted for the purposes of a negative control: two unstained oocytes are visible (white arrows). Scale bars = 20µm.

5.3.2.2 Bovine experiments

As a result of the murine experiments, the overnight hanging drop culture method was used in the bovine experiments in order to encourage formation of a spherical pellet. Culture in collagen was attempted in the first experiment, however, in contrast to the murine pellets, the bovine pellets did not protrude into the collagen. A membrane culture was also attempted; yet, pellets cultured in collagen or on a membrane for the remainder of the culture period were extremely unhealthy, as demonstrated on H&E analysis (Fig 5.9).

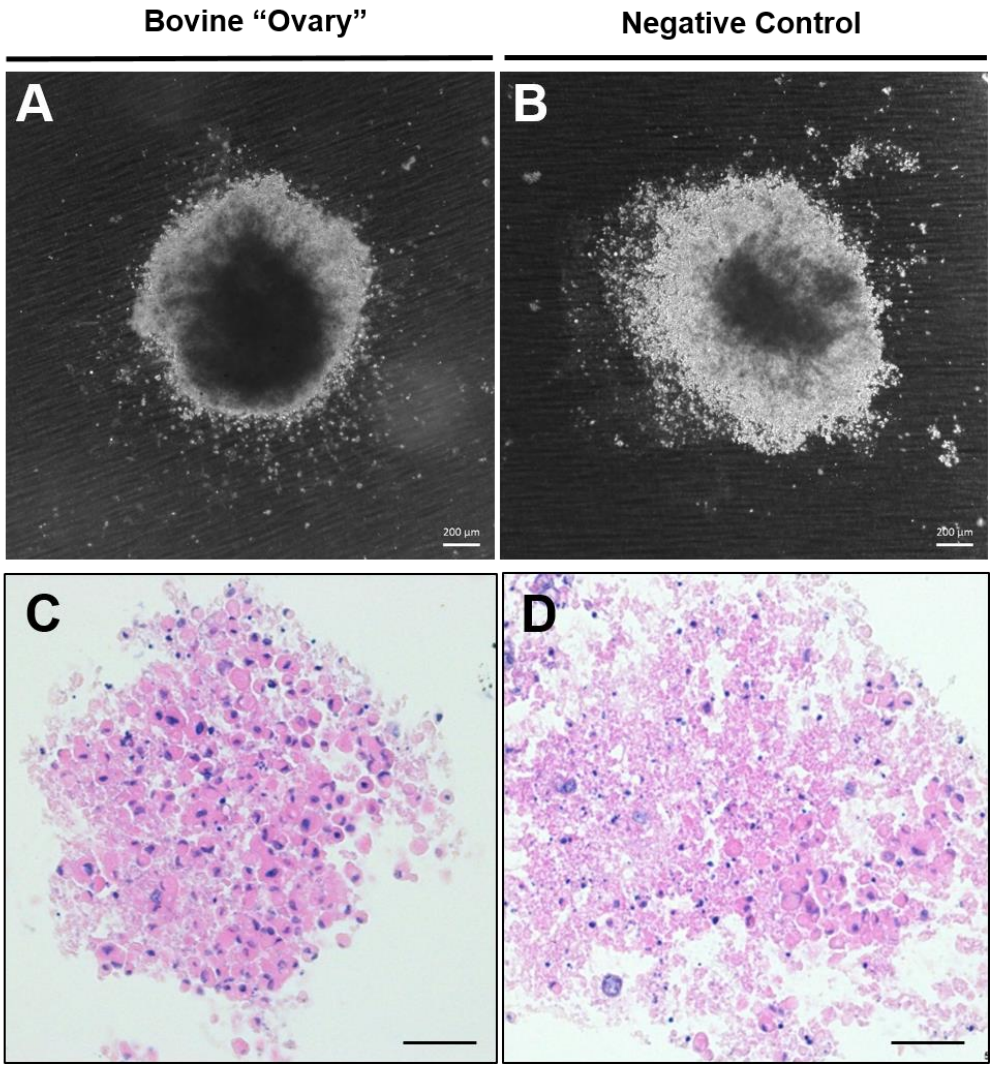


Figure 5.9. Images of a bovine re-aggregated "ovary" and its corresponding negative control. (A, B) Phase contrast images of pellets cultured on membranes demonstrating a rounded structure. Scale bars = 200µm. **(C, D)** H&E analysis revealed very unhealthy structures, with shrunken, pyknotic nuclei and no clear distinction between the two cell populations. Scale bars = 50µm.

A change in culture medium to the more complex and growth factor-enriched OSC culture medium did not improve the health of the pellet. It was difficult to distinguish between the two cell populations on H&E staining alone, but rhodamine labelling of putative OSCs demonstrated that they were evenly dispersed throughout the pellet (Fig. 5.10). However, no morphological changes to the cells (e.g. expansion in size) or follicle formation was observed. The experiment could only be performed twice due to cell availability and time constraints.

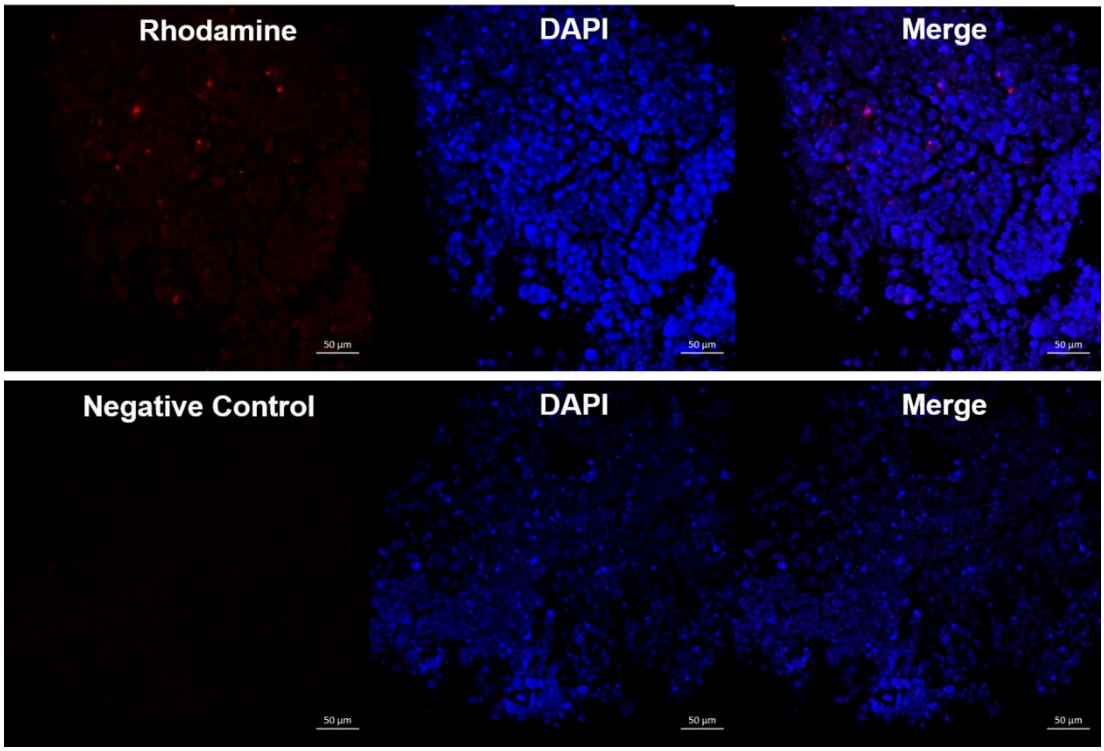


Figure 5.10. Rhodamine-labelled putative OSCs (red) were evenly dispersed throughout the pellet. DAPI (blue) was used as a nuclear counterstain. A somatic cell-only pellet was used as a negative control. Scale bars = 50µm.

5.3.2.3 Human experiments

One human experiment was performed prior to testing the culture system with murine tissue, whereby the pellets were cultured on a membrane for the entire culture period. H&E analysis of these positive pellets revealed larger cells, with expanded cytoplasm, one of which was associated with somatic cells in a primordial follicle-like structure (Fig. 5.11). Such cells were not observed in the negative control.

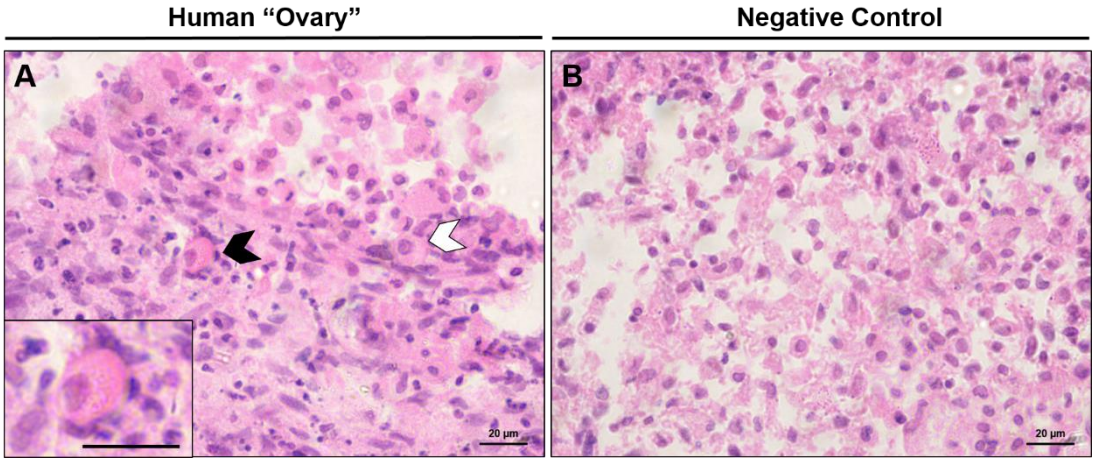


Figure 5.11. H&E analysis of a human re-aggregated "ovary" and its corresponding negative control. (A) Larger cells (white arrow) were seen within the aggregate, with one cell associated with somatic cells in a primordial follicle-like structure (black arrow). A higher power image is shown in the box. **(B)** Negative control aggregate containing human fetal somatic cells only. Scale bars = 20µm.

Further analysis of the tissue to ascertain whether these larger cells expressed the germ cell markers, LIN28 or DAZL, was not possible due to widespread non-specific DAB staining (Fig. 5.12). The reason for this is unclear, but may be due to non-specific binding of the DAB to the PHA used to aggregate the cells. The small number of tissue sections meant that further optimisation of the method was not possible. IF demonstrated a small number of DDX4-positive cells within the pellet but no follicular structures were observed (Fig. 5.13).

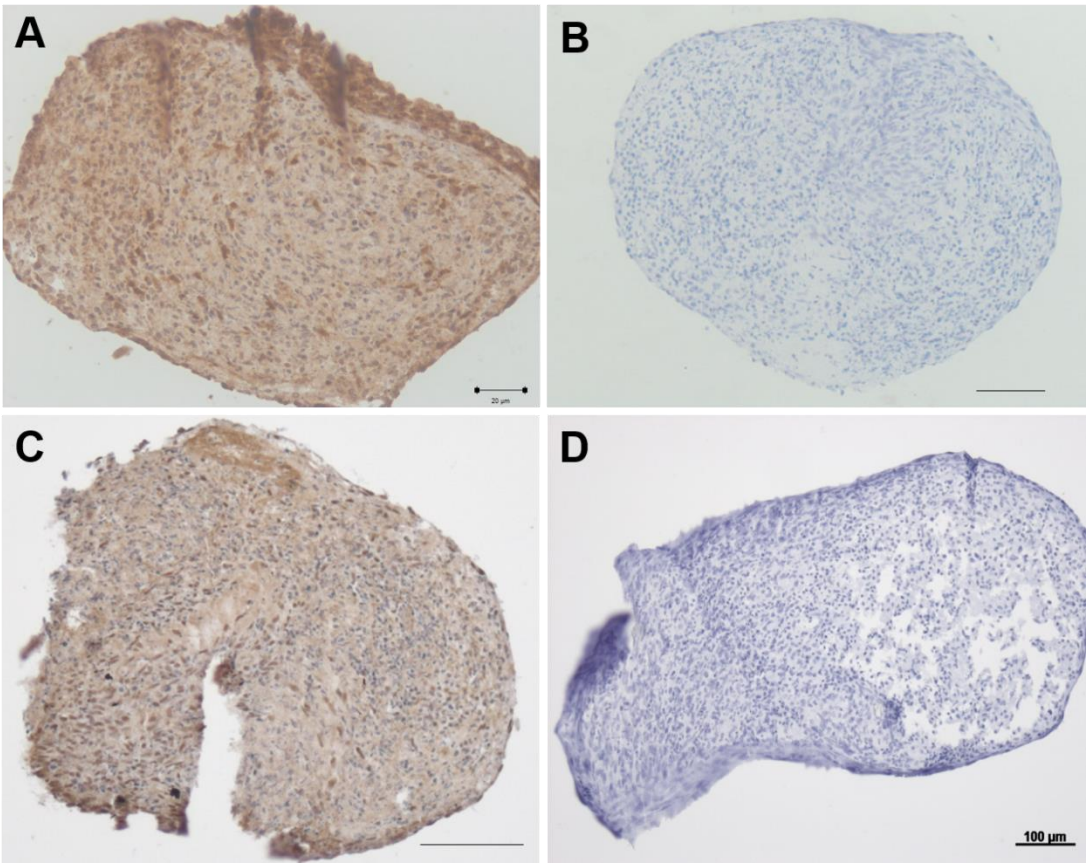


Figure 5.12. Immunohistochemical analysis was uninterpretable for either the presence of LIN28 (A, B) or DAZL (C, D). The primary antibodies were omitted for negative controls **(B, D)**. Scale bars for (A, B) = 20µm. Scale bars for (C, D) = 100µm.

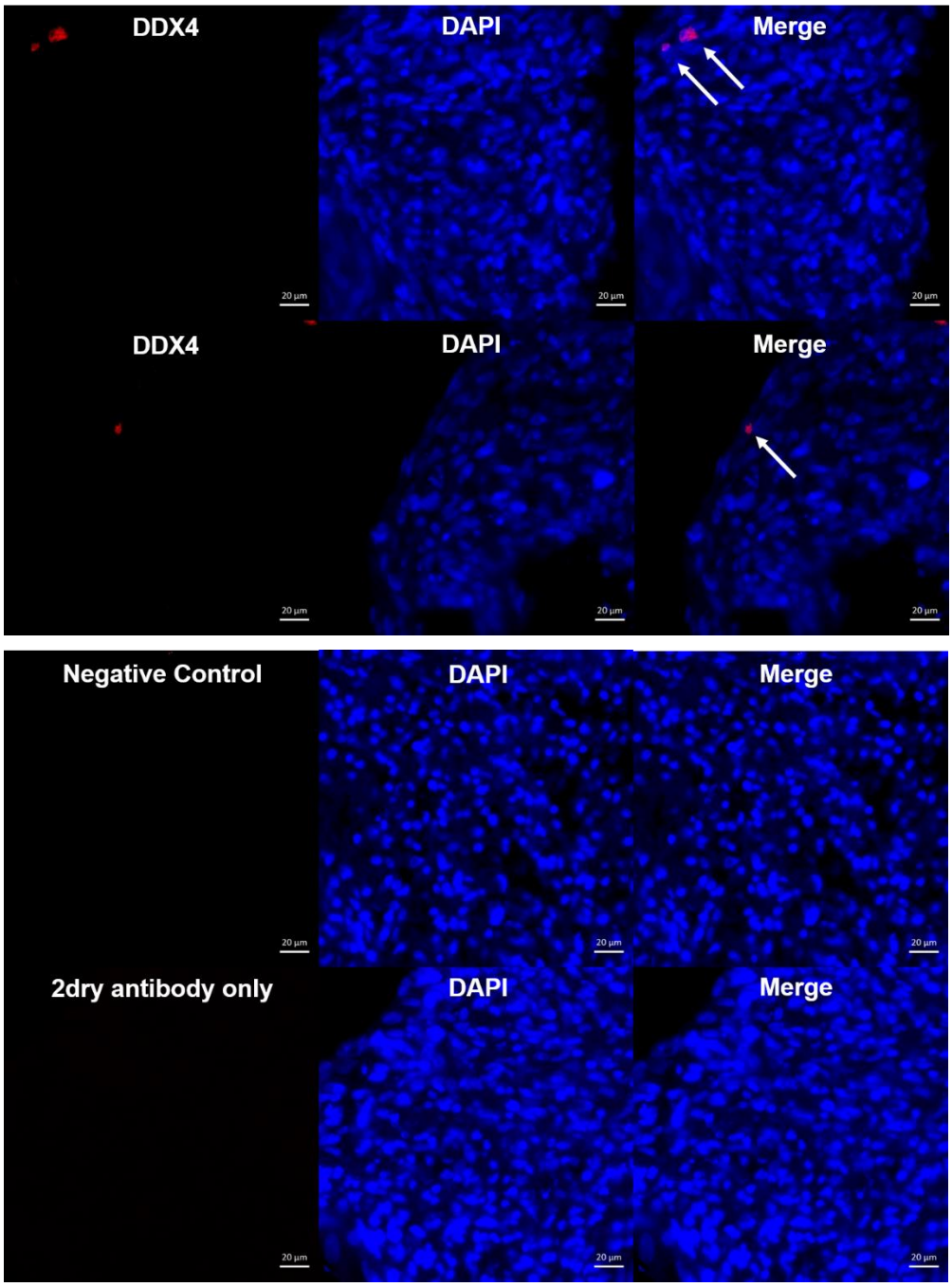


Figure 5.13. A rare population of DDX4-positive cells (red) were detected within a re-aggregated pellet from the first human experiment (white arrows). DAPI (blue) was used as a nuclear counterstain. A somatic cell only pellet and a positive pellet with the primary antibody omitted were used as negative controls. Scale bars = 20µm.

The remaining human experiments were performed following the murine experiments, the results of which informed the decisions regarding the methodologies used. Therefore, hanging drops were used for initial overnight culture to promote a spherical structure and Bouin's was used as a fixative. As seen in the bovine experiments, the human pellets did not protrude into the surrounding collagen and pellets grown on either collagen (Fig. 5.14) or membranes (Fig. 5.15) for the remainder of the culture period were extremely unhealthy, as demonstrated by H&E staining.

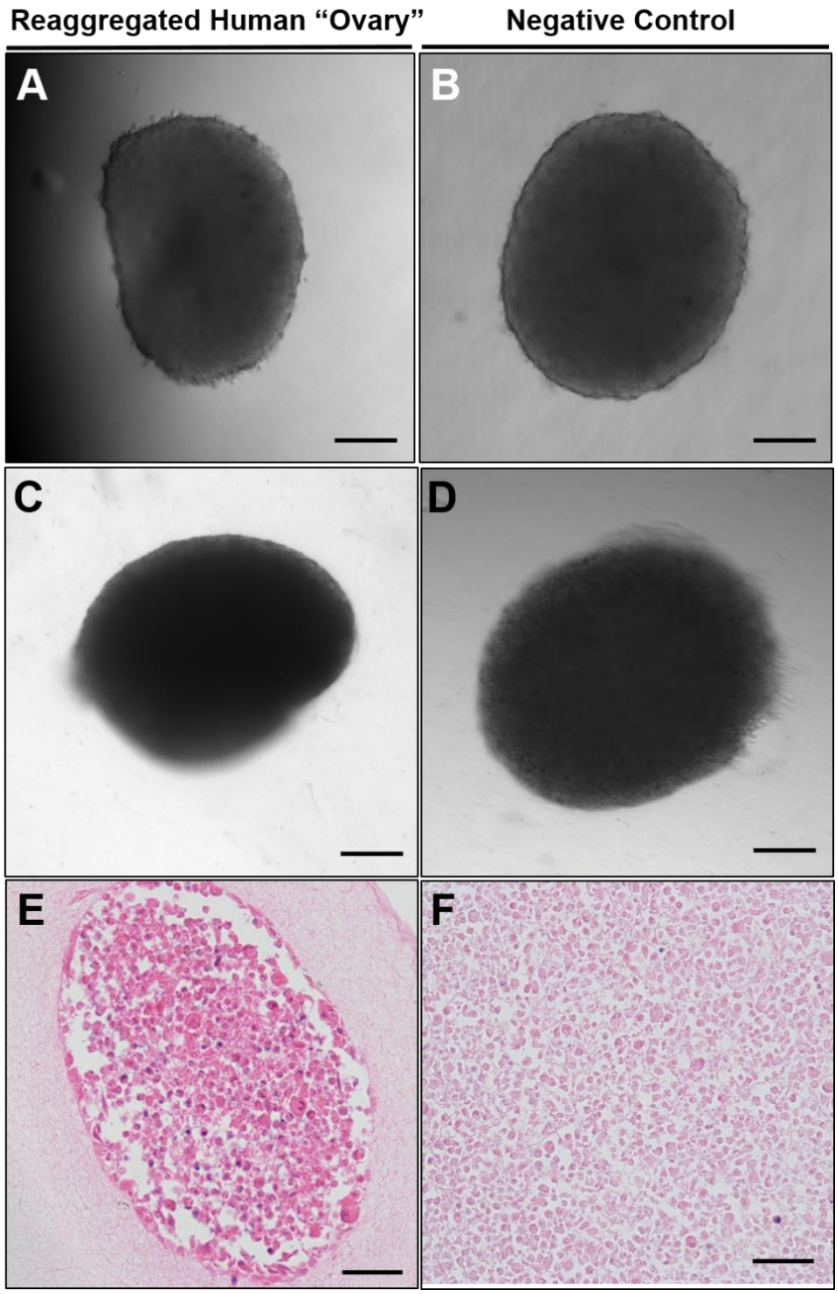


Figure 5.14. Re-aggregated human "ovary" and its corresponding negative control cultured in collagen. (A, B) Bright field images of pellets after 24 hours in hanging drop culture, with a spherical structure seen. Scale bars = 200µm. **(C, D)** Bright field images after 1 week of culture in collagen droplets demonstrating no projection of cells into the collagen. Scale bars = 200µm. **(E, F)** H&E analysis of the pellets demonstrating poor cell viability, especially in the positive pellet. Scale bars = 50µm.

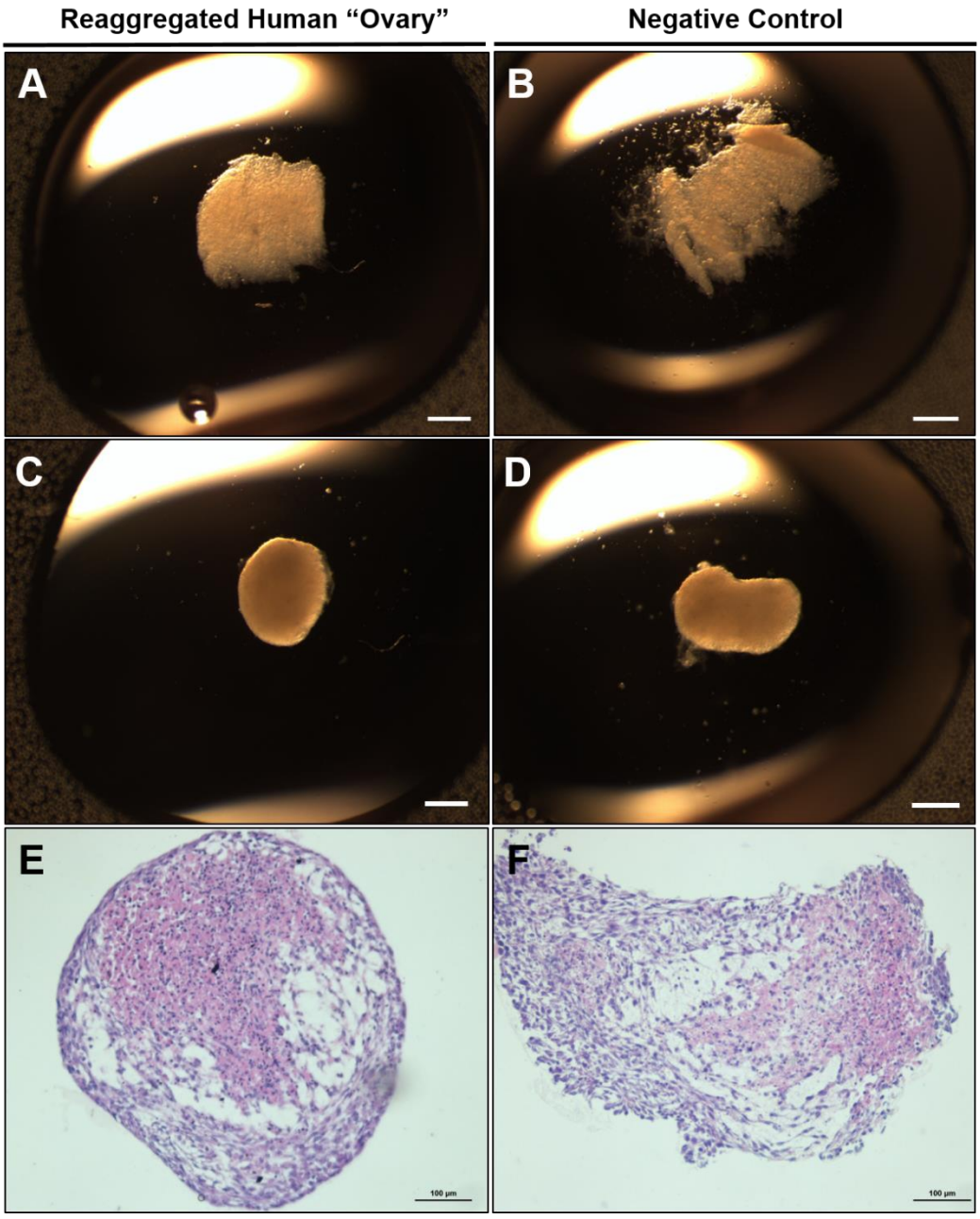


Figure 5.15. Re-aggregated human "ovary" and its corresponding negative control cultured on a membrane. (A, B) Images of pellets immediately after re-aggregation and transfer into hanging drops, with a flat morphology. Scale bars = 500µm. **(C, D)** Images after 24 hours in the hanging drops, demonstrating compaction of the pellet and a spherical structure. Scale bars = 500µm. **(E, F)** H&E analysis of the pellets at the end of the culture period, demonstrating poor cell viability. Scale bars = 100µm.

5.3.2.4 Chimaeric experiment

Given the bovine and human aggregation results, a chimaeric experiment was performed in order to assess whether fetal bovine somatic cells could support *neo*-folliculogenesis with mouse germ cells. No follicles, or recognisable germ cells, were observed in the pellet (Fig. 5.16).

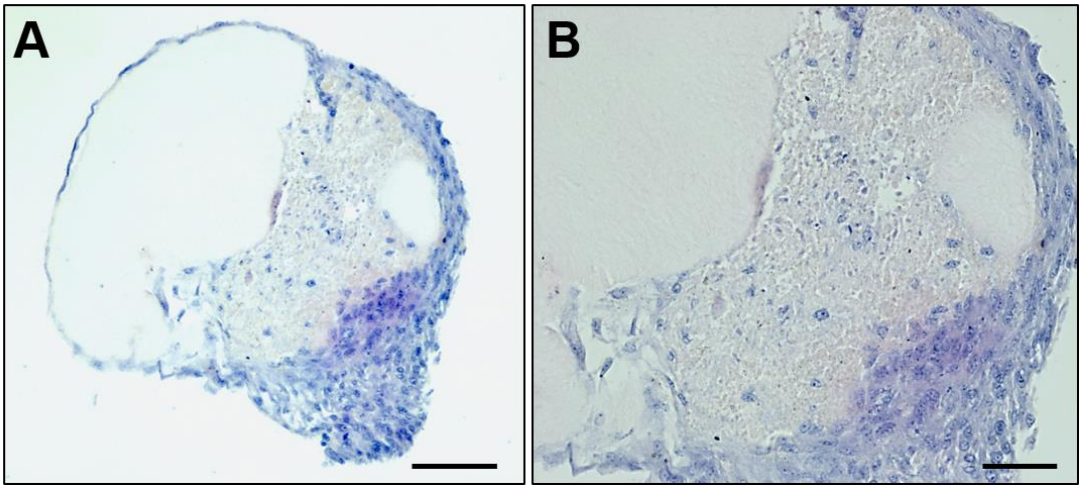


Figure 5.16. Fetal bovine somatic cells did not support folliculogenesis with mouse germ cells. (A) No follicles were observed in the chimaeric pellet. Healthy cells were located around the periphery, whilst the central area was unhealthy and comparatively acellular. Scale bar = 100µm. **(B)** An enlarged image demonstrating the unhealthy central area. Scale bar = 50µm.

5.4 Discussion

The experiments detailed in this Chapter have demonstrated that, rarely, putative human OSCs can form a close association with somatic cells, with some apparently located in primordial follicle-like structures. It was not possible to prove that the OSCs had differentiated into oocytes, with methodological issues contributing to difficulties in data interpretation. Yet, it should be stated that the recapitulation of germ cell development from before the primordial follicle stage to a mature oocyte, entirely *in vitro*, has not been reported in any species and therefore the optimum environment for the support of this pathway remains unknown. As such, selecting the culture conditions for the experiments in this Chapter was a complex and difficult task with many possible variables, including the surrounding supportive cells, the structure of any extracellular support and the composition of the culture medium with regards growth factors etc. The findings described herein therefore represent significant preliminary work towards investigation of the most favourable *in vitro* environment for OSC differentiation.

5.4.1 Injection experiment issues

Several groups have successfully utilised injection techniques to deliver OSCs into whole ovaries (Zou *et al.*, 2009, White *et al.*, 2012, Wolff *et al.*, 2013, Wolff *et al.*, 2014, Zhou *et al.*, 2014, Xiong *et al.*, 2015, Lu *et al.*, 2016) or ovarian cortical pieces (White *et al.*, 2012), and have demonstrated the ability of the cells to undergo oogenesis. However, all of the investigators used the technique in *in vivo* approaches. The decision to focus on *in vitro* culture systems and the proficiency of Prof. Telfer's group in *in vitro* follicle culture (Telfer *et al.*, 2008, McLaughlin and Telfer, 2010) led to the novel approach of injecting putative OSCs into ovarian cortical pieces and subsequent *in vitro* tissue culture. Despite being theoretically straightforward, several methodological difficulties were experienced which resulted in inconclusive results.

Firstly, the injections were technically challenging: the use of small cortical fragments is crucial to the success of the culture system (Telfer and McLaughlin, 2011) and the delivery of OSCs into these small fragments was difficult. Cell losses occurred via unintended extrusion of the cell suspension into the surrounding medium and, additionally, areas of dense stroma in the tissue impeded the injection of the cells. In

an attempt to improve cell delivery, blue dye was used to aid visualisation of the cell suspension and the protocol was altered so that cells were injected into larger tissue pieces, which were then cut into smaller fragments; however, neither of these modifications was effective. As a result, the number of cells estimated to be injected was much less than that reported by White *et al*., who injected approximately 5 - 6 times more cells into the same volume of tissue (White *et al.*, 2012). Moreover, the manipulation of the tissue for injection and the insertion of the needle often resulted in visible damage to the structural integrity of the tissue, especially in the human experiments. Thus a combination of low putative OSC numbers and inadequate somatic cell environment may have prevented the process of *neo*-oogenesis.

A crucial technical issue concerned the IHC analysis of the injected tissue. Despite many alterations to the IHC protocol, with a focus on increased blocking processes, non-specific binding of the GFP and RFP antibody to some oocytes was observed in all treatment groups. Small GFP-positive cells in association with somatic cells and follicular structures were only observed in putative OSC-injected tissues; nevertheless, due to the non-specific staining, robust interpretation of the results in relation to assessing the developmental potential of injected OSCs was not possible.

Finally, it was observed that transduced cells did not appear to proliferate as quickly during *in vitro* culture as non-transduced cells (see Chapter 3); as such, lentiviral transduction was considered to be detrimental to the health of the cells. It is therefore possible that, even if the cells were delivered efficiently into the tissue, they were less likely to have the capacity to undergo *neo*-oogenesis. As a result of these difficulties, a second method was undertaken: the creation of artificial ovaries.

5.4.2 Artificial "ovary" experiment issues

The creation of an artificial ovary was postulated to have two main benefits over the injection experiment: (1) it would allow the combination of putative OSC and somatic cells to occur in a more controlled manner, and (2) fluorescent labelling of the putative OSCs was not essential as any oocytes observed in the "ovary" must have been OSC-derived. Re-aggregated "ovaries" are a well-recognised technique for analysing differing aspects of oocyte and follicle development; for example, such experiments have revealed that XY germ cells can mature in an XX somatic cell environment, but not vice versa (O and Baker, 1978); that oocyte-somatic cell interactions are

evolutionarily conserved as rat ovarian somatic cells can support the normal development of mouse germ cells, and vice versa (Eppig and Wigglesworth, 2000); that the oocyte controls the rate of follicular growth in mice (Eppig *et al.*, 2002); and that the gap junction protein, connexin37, is essential for oocytes to communicate with granulosa cells (Gittens and Kidder, 2005). Similar techniques have been used in OSC research by two groups: Pacchiarotti *et al*. demonstrated that aggregates of neonatal granulosa cells and mouse OSCs generated primordial follicle-like structures when cultured in hanging drops overnight (Pacchiarotti *et al.*, 2010), whilst White *et al*. reported comparable results, with 24 hour cultures of aggregates containing dissociated adult human ovarian cortex and human OSCs on membranes also generating follicle-like structures (White *et al.*, 2012). The *in vitro* culture was not extended past 24 (Pacchiarotti *et al.*, 2010) or 72 (White *et al.*, 2012) hours and the developmental competency of OLCs within the follicle-like structures was not tested. A further advantage of the technique was, therefore, that it had already been validated for use with OSCs *in vitro*.

An initial attempt at creating human artificial "ovaries" and culturing them entirely on membranes revealed some technical difficulties: handling of the pellet immediately after centrifugation often led to its disintegration, and an inadequately spherical structure was formed. It was therefore decided to use mouse artificial "ovaries" as a model for the technique in order to optimise it prior to further experimentation with the less freely available putative OSCs. The optimal technique, which supported the formation of healthy immature murine follicles that maintained their expression of DDX4, comprised an overnight hanging drop culture and subsequent encapsulation in collagen for the remainder of the culture period. Hanging drop culture (or inverted drop culture) has previously been reported to be a valuable *in vitro* culture system for both follicles (Wycherley *et al.*, 2004, Nation and Selwood, 2009) and fetal ovaries (Jorgensen *et al.*, 2015). Both mouse (Wycherley *et al.*, 2004) and marsupial (Nation and Selwood, 2009) follicles demonstrated improved growth and viability using this approach compared to upright cultures and short term culture of human fetal ovaries supported germ cell proliferation (Jorgensen *et al.*, 2015). In our experiments, the hanging drop was found to be very effective at promoting the formation of a spherical "ovary" from the initial flat, disc-shaped pellet; this, coupled with encapsulation in collagen, allowed the pellet to maintain a 3D structure throughout the culture period.

This is in contrast to the pellets cultured on membranes, which did not form a spherical structure. This is an important aspect of the culture system as a 3D structure is more comparable to the normal structure of an ovary and, compared to a two-dimensional (2D) approach, allows increased cell-to-cell interaction between somatic cells and putative OSCs for possible folliculogenesis (Fig. 5.17). It is well-established that 3D culture systems are superior at mimicking the *in vivo* environment than 2D approaches (Cukierman *et al.*, 2001) and, although 2D systems can support oocyte maturation from primordial follicles in mice (Eppig and O'Brien, 1996, Cortvrindt *et al.*, 1996, O'Brien *et al.*, 2003), mouse follicles lose their spherical follicular structure during the culture period, with the growth of granulosa cells out through the basement membrane of the follicle (Cortvrindt *et al.*, 1996). It is possible that this loss of 3D structure has contributed to the inability to recapitulate such experiments in larger animals with more prolonged follicle development.

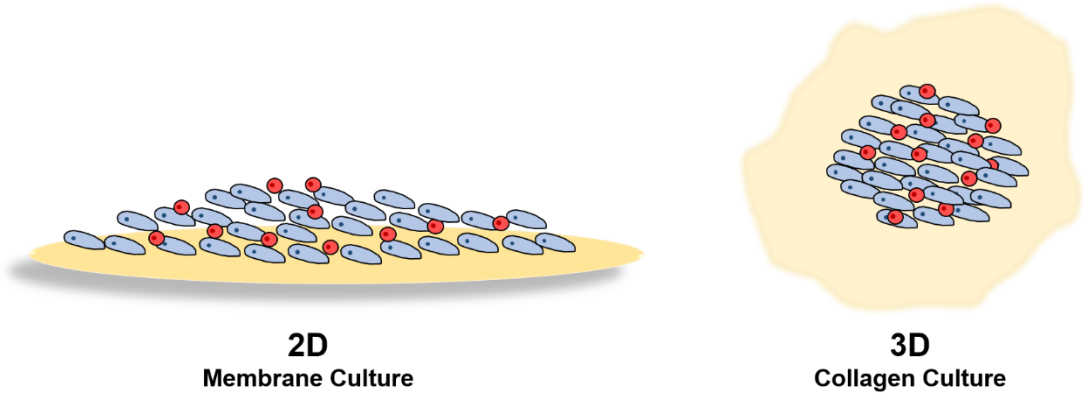


Figure 5.17. Three-dimensional (3D) culture systems, such as encapsulation in collagen, mimic *in vivo* circumstances better than two-dimensional (2D) systems, with increased cell-to-cell contact.

Collagen is only one possible option as an ECM: other materials such as alginate, hyaluronic hydrogels and synthetic matrices have all been used in 3D *in vitro* culture (West *et al.*, 2007, Belli *et al.*, 2012). However, collagen was selected as it is an important component of both the basement membrane of the follicle and the surrounding stroma (Rodgers *et al.*, 2003) and is therefore a physiologically relevant, biocompatible material. Moreover, its use as an ECM for successful support of *in vitro* follicle development has been validated previously in several species, including mice (Torrance *et al.*, 1989), pigs (Hirao *et al.*, 1994), buffalo (Sharma *et al.*, 2009) and humans (Abir *et al.*, 1999). Not only did collagen provide mechanical support to the

mouse pellets, but it also appeared to support cell proliferation, with visible protrusion of cells growing into it. This system supported healthy follicular development for up to two weeks, although cell death was detectable, with pyknotic nuclei concentrated in the centre of the pellet. Furthermore, germ cells were restricted to the periphery of the pellet, suggesting that cells in closer proximity to the surrounding culture medium had improved survival.

Yet, despite optimisation of the culture system in mice, the application of the technique to bovine and human putative OSCs was not successful. One issue may have been the ratios of somatic cells to putative OSCs used in the experiments: the methodologies of previously published literature did not state the successfully utilised ratios of germ cells to somatic cells (O and Baker, 1978, Eppig and Wigglesworth, 2000, Eppig *et al*. 2002, Gittens and Kidder, 2005), resulting in ratios being chosen principally on cell availability. Although ratios of 10 – 50 somatic cells to 1 germ cell were effective in the mouse experiments, it may be that much higher ratios of somatic cells to putative OSCs are required for successful folliculogenesis to occur. Furthermore, in contrast to previous OSC studies using neonatal (Pacchiarotti *et al.*, 2010) and adult (White *et al.*, 2012) somatic cells, our approach utilised fetal somatic cells. This strategy was chosen in the anticipation that it might provide a more developmental stage-appropriate somatic cell environment for the earliest steps of follicular assembly, with the cells possessing more plasticity than those derived from post-natal ovaries. The importance of the use of somatic cells at the correct developmental stage has been demonstrated by Hayashi *et al*., who successfully induced the development of mature, fertilisable oocytes by incorporating fetal gonadal somatic cells mouse into aggregates with ESC- and iPSC-derived PGCLCs (Hayashi *et al.*, 2012).

The observation of possible primordial follicle-like structures was observed in only one experiment, where human aggregates were cultured on membrane for the entire culture period. Only morphological assessment of the structures was possible, with IHC providing uninterpretable results. IF did demonstrate that a small population of DDX4-positive cells could be detected in the same human pellet, which was not present in the negative controls, although they were not larger than the surrounding cells and no follicular structures were identified. The sparsity of these cells indicates that either high numbers of OSCs are lost during the technique (for example, during the pellet formation or through cell death) or that not all OSCs are expressing DDX4.

The former hypothesis is supported by the finding of high levels of mouse germ cell loss in the murine experiments: it may be that the re-aggregation process reduces germ cell viability or that the somatic cells can only support a certain number of germ cells. The latter hypothesis is supported by the finding of inconsistent DDX4 protein expression in cultured putative OSCs (as described in Chapter 4).

However, none of the other experiments, which utilised modifications tested in the mouse model, generated such structures, with extremely poor cell health observed. Cell viability was not improved by exposure to more growth factors within the OSC culture medium. Moreover, bovine and human pellets did not appear to utilise collagen as a ECM in the same manner as observed in the murine experiments, with few, or no, cellular projections seen protruding from the pellets. A chimaeric experiment, performed to test whether fetal bovine somatic cells could support folliculogenesis with mouse germ cells, resulted in no follicle formation. It is therefore possible that *neo*-oogenesis could not be confirmed because: (a) cells did not thrive within the culture systems, (b) the fetal somatic cells may not have been able to support differentiation of OSCs and/or (c) the putative OSCs may not be genuine OSCs.

Unfortunately, there were a great deal of variables in these experiments, thus it is difficult to determine which aspect(s) required alteration. Furthermore, the number of bovine and human experiments performed was restricted by cell availability, thereby constraining the ability to optimise the culture system. Due to time and tissue constraints, further experimentation on *neo*-oogenesis was outwith the scope of the research.

5.4.3 Summary of this chapter

In vitro culture systems are extremely useful basic science tools in ovarian biology, providing a controllable environment for the examination of folliculogenesis and oocyte development. Two novel *in vitro* culture systems for the support of OSC differentiation, adapted from previously validated *in vivo* approaches, were utilised; however, the approaches provided inconclusive results regarding the ability of the putative bovine and human OSCs to generate new oocytes. There are two principal reasons that may account for this finding: (1) the putative OSCs are not capable of undergoing *neo*-oogenesis and (2) the culture systems cannot support OSC

differentiation. Further optimisation of the culture systems may allow a more definitive assertion regarding the oogenesis potential of the putative OSCs.

Chapter 6

The Potential of Putative OSCs as a Germ Cell Model

6.1 Introduction

6.1.1 Stem cells as models for germ cell development

As discussed in Chapter 5, OSCs have the potential to be an invaluable tool in basic science, as their *in vitro* culture would allow investigation of germ cell development, interaction with somatic cells, and differentiation into oocytes. To date, much of our knowledge of human female germ cell development has been inferred from rodent models; however, variations in the process between species mean that a human-specific model, or at least a large animal model that may be more translational to humans, is necessary for a more accurate understanding (Jung and Kee, 2015). Recent developments have demonstrated that ESCs and iPSCs are a promising novel way of exploring germ cell development: the induction of such cells to form female mouse PGCLCs *in vitro*, which were shown to form viable offspring (Hayashi *et al.*, 2012), will allow closer examination of the earliest stages of germ cell development. Furthermore, the ability to perform these experiments *in vitro* provides a more convenient, and controllable, system than *in vivo* strategies. Yet, once again, this research utilises the mouse model and does not address human germ cell development directly.

Human ESCs and iPSCs can be induced *in vitro* to form EBs (Aflatoonian *et al.*, 2009) and PGCLCs (Kee *et al.*, 2009, Irie *et al.*, 2015, Sugawa *et al.*, 2015). Resultant oocyte-like and primordial follicle-like structures have been observed by morphology; however, zona pellucida was never detected in these structures, thus uncertainties remain regarding whether mature oocytes were produced (Aflatoonian *et al.*, 2009). Nevertheless, hESC studies have elucidated a critical aspect of early PGC development: the transcription factor, SOX17, has been shown to be the earliest marker of human PGCLCs and is essential for PGCLC specification (Irie *et al.*, 2015). It is possible it determines germ cell fate via its downstream stimulatory effects on PRDM1, which is known to be essential for PGC development (Vincent *et al.*, 2005, Ohinata *et al.*, 2009). A *SOX17* knock-out hESC line did not form PGCLCs and lacked expression of *PRDM1*, in contrast to the wild-type cells (Irie *et al.*, 2015). This is an example of species dimorphism, as Sox17 has no role in mouse PGC specification (Hara *et al.*, 2009), thus emphasising the need for a human-specific model of germ cell development.

In both humans and mice, ESCs and iPSCs have been directed to differentiate into PGCLCs by altering the culture medium to contain key regulators of PGC development. A "pre-induction" of naïve ESCs to primed epiblast-like cells (EpiLCs) has been performed using activin A and bFGF (Hayashi *et al.*, 2012, Irie *et al.*, 2015), whilst subsequent formation of PGCLCs has been induced by the presence of BMPs, including BMP4 (Aflatoonian *et al.*, 2009, Kee *et al.*, 2009, Hayashi *et al.*, 2012, Irie *et al.*, 2015, Sugawa *et al.*, 2015). RA has also been utilised as an induction agent in hESCs (Aflatoonian *et al.*, 2009).

As OSCs are presumed to be located further along the differentiation pathway than totipotent ESCs, they may provide an alternative germ cell model that would require less manipulation to create *in vitro* PGCLCs. If OSCs are to be used in this regard, they must demonstrate similar responses to key regulators of PGC development as that seen *in vivo*. A key aspect to explore would be the expression of meiosis-related genes as (a) meiosis is an essential aspect of oocyte formation, and (b) germ cells are the only cell known to be capable of this process; thus, the detection of meiotic genes could provide preliminary evidence that the OSCs may be undergoing differentiation. To date, only one study has been performed on OSCs investigating this possible application (Park *et al.*, 2013). Park *et al*. demonstrated that mouse OSCs express BMP receptors and respond to BMP4 in the same manner as PGCs, with upregulation of genes involved in meiotic initiation, namely *Stra8*, *Msx1* and *Msx2* (Park *et al.*, 2013). In addition, BMP4 treatment resulted in an increase in the appearance of OLCs, in a dose-dependent manner. Given their critical roles in PGC development, meiotic entry and in the stimulation of pluripotent stem cell and OSC differentiation, this Chapter will focus on the effects of two major regulators, BMP4 and RA, on putative bovine and human OSCs in order to assess their potential as a germ cell model.

6.1.2 Bone morphogenetic protein-4 (BMP4)

BMP4 is a member of the TGFβ1 family of growth factors, which has many essential roles in organogenesis. The first known function of the BMP family of cytokines was in the induction of formation of new bone, hence their name; however, they are now known to be involved in proliferation, differentiation and apoptosis within many divergent biological systems, including skeletal muscle development, the formation of

adipose tissue, eye development and the regulation of iron homeostasis (Hogan, 1996, Katagiri and Watabe, 2016). Most pertinent to this research are their roles in germ cell development: Bmp2, 4 and 8b have all been demonstrated to be critical in murine PGC specification and proliferation (Lawson *et al.*, 1999, Ying *et al.*, 2000, Ying and Zhao, 2001), with Bmp4 homozygous null mutants being the most severely affected with regards to PGC numbers (Lawson *et al.*, 1999). In humans, data are more limited, however, BMP receptors are expressed by germ cells and BMP4 has been found to stimulate PGC apoptosis in the human fetal ovary (Childs *et al.*, 2010).

BMP4 is activated by cleavage of its inactive precursor by furin (a proprotein convertase enzyme (Nelsen and Christian, 2009)) to form mature, monomeric carboxy-terminal proteins which can dimerise to produce a biologically active ligand (Katagiri and Watabe, 2016). The active protein acts through serine-threonine kinase transmembrane receptors, binding initially to a type II receptor, which then complexes with a type I receptor and results in phosphorylation of intracellular signaling proteins, called SMAD proteins (Hogan, 1996, Katagiri and Watabe, 2016; Fig. 6.1). SMADs are transcription factors, with SMAD1, SMAD5 and SMAD8 being receptor-regulated SMADs that, once phosphorylated, form a complex with SMAD4 (Katagiri and Watabe, 2016). SMAD4 enables translocation of the complex to the nucleus to elicit downstream effects on BMP4-response genes.

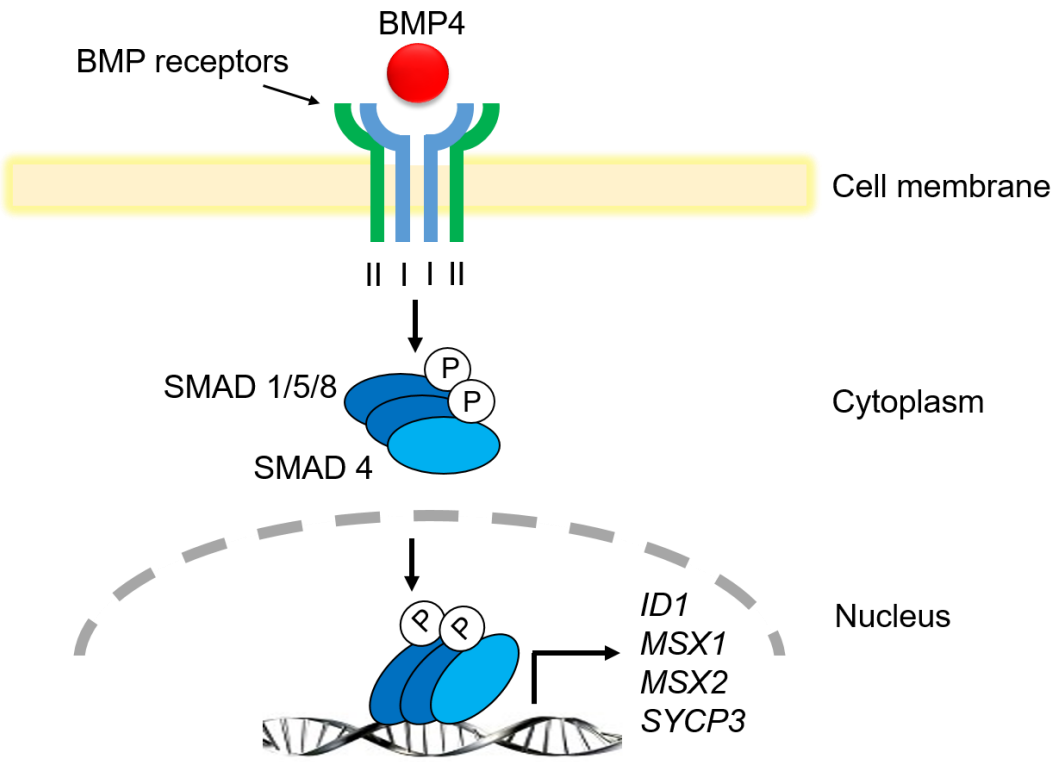


Figure 6.1. Schematic of the BMP signalling pathway. BMP4 binds to Type II BMP receptors (green), which form a complex with Type I receptors (blue). This stimulates phosphorylation (P) of intracellular SMAD proteins, which move into the nucleus and stimulate transcription of BMP4-response genes, including *ID1*, *MSX1*, *MSX2* and *SYCP3*. Adapted from Katagiri and Watabe, 2016.

Four examples of BMP4-response genes are Muscle segment homeobox 1 (*MSX1*), *MSX2*, *SYCP3* and *ID1*. *MSX1* and *2* have been demonstrated to have a role in meiosis initiation in the mouse, with reduced numbers of meiotic cells seen in double *Msx1*/*2* homozygous null mice (Le Bouffant *et al.*, 2011). This deficit is apparently "rescued", however, if either *Msx1* or *2* are present, with single *Msx1* or *Msx2* null mice exhibiting no change in meiotic cell numbers (Le Bouffant *et al.*, 2011). The same study reported that Bmp4 treatment of mouse fetal ovaries upregulates *Msx1* and *2* expression (Le Bouffant *et al.*, 2011). In humans, *MSX2* appears to mediate BMP4's pro-apoptotic effects, both in the human fetal ovary (Childs *et al.*, 2010) and in other cell types, including an embryonal carcinoma cell line (Marazzi *et al.*, 1997). SYCP3 is a

component of the synaptonemal complex which is required for the correct pairing of homologous chromosomes by chiasmata during zygotene (Yuan *et al.*, 2002): as such, it can be considered a marker of meiotic entry. It is also upregulated in response to BMP4, with BMP4 treatment of hESCs resulting in EB formation and concurrent upregulation of *SYCP3* expression (Kee *et al.*, 2006). Lastly, unpublished work from Prof. Anderson's group has demonstrated that *ID1* expression is stimulated by BMP4 treatment in human fetal ovaries: this has been supported by similar findings in mouse embryonic gonads (Dudley *et al.*, 2007) and other cell types, including mouse ESCs (Hollnagel *et al.*, 1999) and human pulmonary arterial smooth muscle cells (Yang *et al.*, 2013).

6.1.3 Retinoic acid (RA)

RA, an active derivative of Vitamin A, is an important morphogen within the developing embryo, with crucial roles in the correct development and differentiation of various tissues, including the brain, limbs and neural tube (Rhinn and Dolle, 2012). Its role in germ cell development is also well-established: murine studies have revealed that RA is essential for meiotic entry of PGCs as antagonism of RA receptors (RARs) results in female PGCs failing to initiate meiosis (Bowles *et al.*, 2006, Koubova *et al.*, 2006). Human data have revealed by IHC that germ cells are the principal target of RA signaling in the fetal ovary, with two RARs (RARα and RARβ) and one retinoid receptor (RXRα) predominantly localised to the germ cells (Childs *et al.*, 2011). In addition, two separate *in vitro* culture systems have demonstrated that RA treatment of human fetal ovaries significantly increases the number of meiotic germ cells present, as identified by the protein expression of doublesex and mab-3 related transcription factor 1 (DMRT1; a regulator of meiosis) (Jorgensen *et al.*, 2015) and H2A histone family, member X (ɣH2AX; a marker of the double-strand breaks (DSBs) in DNA which allow recombination during meiosis) (Kuo and Yang, 2008, Le Bouffant *et al.*, 2010, Jorgensen *et al.*, 2015).

RA is produced by two successive oxidation steps: firstly, retinol is oxidised to retinaldehyde by retinol dehydrogenases, then retinaldehyde is converted to RA by retinaldehyde dehydrogenases (Rhinn and Dolle, 2012). Levels of RA are controlled

by the presence of RA-inactivating cytochrome p450, 26 (CYP26) enzymes (Rhinn and Dolle, 2012). In mice, *Cyp26b1* is widely expressed by the somatic cell compartment of the fetal testes, in particular the Sertoli cells, and thus prevents premature entry of male germ cells into meiosis (Bowles *et al.*, 2006), although this phenomenon does not appear to be conserved in humans (Childs *et al.*, 2011). RA stimulates the nuclear receptors, RARα, RARβ and RARɣ, which form heterodimers with nuclear retinoid X receptors (RXRα, RXRβ and RXRɣ) (Bowles and Koopman, 2007, Rhinn and Dolle, 2012; Fig. 6.2). These heterodimers can bind with the RA-response element (RARE) of RA-responsive genes (Bowles and Koopman, 2007). RA-response genes of interest in the ovary are related to meiosis, with murine studies demonstrating upregulation of *Msx1*, *Sycp3* and *Stra8* in fetal ovaries or isolated PGCs in response to RA (Bowles *et al.*, 2006, Koubova *et al.*, 2006, Le Bouffant *et al.*, 2011, Tedesco *et al.*, 2013). One study demonstrated this was accompanied by downregulation of the pluripotency gene, *Pou5f1* (Bowles *et al.*, 2006). RA treatment of human fetal testis and ovary stimulates expression of *STRA8* (Le Bouffant *et al.*, 2010, Childs *et al.*, 2011), but is not associated with a significant change in *SYCP1* in the ovary (Le Bouffant *et al.*, 2010) or *SYCP3* expression in the testis (Childs *et al.*, 2011).

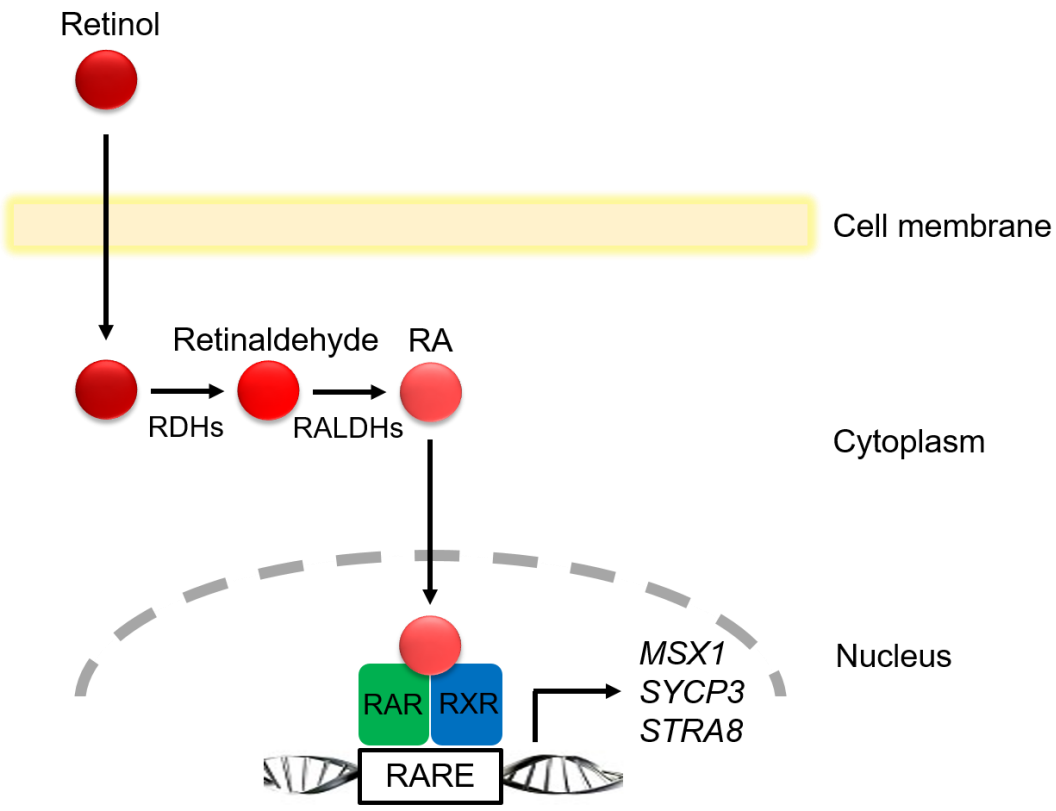


Figure 6.2. Schematic of retinoic acid signalling. Oxidation by retinol dehydrogenases (RDHs) and retinaldehyde dehydrogenases (RALDHs) converts retinol to retinoic acid (RA). RA then binds to nuclear receptors (RAR) which heterodimerise with retinoid X receptors (RXR). These receptors then bind with the RA-response element (RARE) of RA-responsive genes, eliciting their upregulation. RA-response genes include *MSX1*, *SYCP3* and *STRA8*. Adapted from Rhinn and Dolle, 2012.

6.1.4 The role of DAZL in meiosis

An alternative method for the enhanced induction of hESCs and iPSCs has been reported, utilising plasmids to generate overexpression of DAZL within the stem cells (Medrano *et al.*, 2012). Increased DAZL expression resulted in augmented differentiation to PGCLCs and expression of meiotic markers compared with non-transfected cells. DAZL, along with DAZ and BOLL, is a member of the DAZ family of RNA binding proteins. Knock-out mouse studies have demonstrated its essential

role in gametogenesis, with female homozygous null mutants completely lacking in germ cells (Ruggiu *et al.*, 1997). In humans, analysis of single nucleotide polymorphisms (SNPs) of the *DAZL* gene has revealed a correlation between some SNPs and an earlier age of POI (Tung *et al.*, 2006). Specifically, DAZL appears to be a critical requirement for PGC entry into meiosis: following *Dazl* knock-out studies in mice, it has been postulated that intrinsic Dazl expression converts mouse PGCs into meiosis-competent germ cells that are able to respond to extrinsic RA and enter meiosis (Lin *et al.*, 2008). In human fetal ovaries, DAZL is located in central "nests" and expression markedly increases in the second trimester, during the time of meiotic initiation (Anderson *et al.*, 2007, He *et al.*, 2013). This is accompanied by a switch in intracellular location, from the nucleus to the cytoplasm (Anderson *et al.*, 2007). Research has demonstrated that this meiotic role may be linked to *Sycp3* expression: in male *Dazl* null mutant mice, Sycp3 expression is decreased at the protein level and complete synaptonemal complexes are not detected, indicating that Dazl is required for effective translation of *Sycp3* mRNA (Saunders *et al.*, 2003, Reynolds *et al.*, 2007). In humans, overexpression of the DAZL gene in ESCs using plasmids has resulted in increased SYCP3 expression (Kee *et al.*, 2009); however, female PGCs have not been investigated.

6.1.5 Aims of this chapter

The overarching aim of the research detailed in this Chapter was to ascertain whether putative bovine and human OSCs had potential for use as a model for germ cell development. Although mouse OSC research has shown promise in this regard, studies using human OSCs, or OSCs from larger animal models, are currently lacking and there has been no research examining the response of the cells to RA. Therefore, the primary aim was to examine the response of the bovine and human cells to BMP4 and/or RA, by analysing the expression of BMP4- and RA-response genes, including *ID1*, *MSX1*, *MSX2* and *SYCP3*. The secondary aim was to overexpress DAZL in the putative OSCs to explore the effect this may have on cell differentiation and the expression of meiosis-related genes.

6.2 Materials and Methods

6.2.1 BMP4 and/or RA treatment experiments

6.2.1.1 Cell culture and treatments

Putative bovine OSCs (Cell Line 4, Population B, varying passages), putative human OSCs (Patient 1, Population B, varying passages) and fetal bovine somatic cells (P3) were cultured in 12 well plates in OSC culture medium, which included FBS, until approximately 80% confluent. Serum contains a variety of proteins, including BMP4 (Kodaira *et al.*, 2006, Herrera and Inman, 2009), therefore, the cells were serum-starved for 24 hours prior to treatment in order to remove this confounding factor from the experiment. As LIF prevents differentiation of stem cells (Hirai *et al.*, 2011) and the aim of the experiment was to analyse the effect of pharmacological agents on cell differentiation, LIF was also omitted from the culture medium. This was performed by simply replacing the culture medium with OSC culture medium containing no FBS and LIF. The following day, individual wells were allocated to one of four treatment groups (Table 6.1). BMP4 (recombinant human BMP4; Life Technologies), all-trans RA (Sigma-Aldrich) and their diluents (4mM HCl containing 0.1% (w/v) BSA and DMSO respectively) were utilised. The concentrations of BMP4 and RA were chosen as they had previously been used by Prof. Anderson's group in human fetal gonad research (Childs *et al.*, 2010, Childs *et al.*, 2011) and have been successfully utilised in ESC (Kee *et al.*, 2006, Sugawa *et al.*, 2015) and OSC (Park *et al.*, 2013) induction.

All treatments were added to 1ml of OSC culture medium with FBS and LIF omitted. The cells were washed twice with DPBS and the treated culture medium was added to the appropriate wells. After 24 hours of treatment, cells were trypsinised with 0.25% (v/v) trypsin-EDTA, centrifuged at 800 x g for 5 mins at room temperature and resuspended in 350µl RLT buffer containing 1% (v/v) β-ME. Samples were then analysed for gene expression by qRT-PCR experiments.

Table 6.1. Details of the treatments groups used in the germ cell model experiments. The diluent for BMP4 was 4mM HCl + 0.1% BSA and the diluent for RA was DMSO.

Treatment Group	Treatment	Final Concentration
BMP4 only	BMP4	100ng/ml
	DMSO	0.0001% (v/v)
RA only	RA	1.0µM
	4mM HCl + 0.1% BSA	1% (v/v)
BMP4 and RA	BMP4	100ng/ml
	RA	1.0µM
Control	DMSO	0.0001% (v/v)
	4mM HCl + 0.1% BSA	1% (v/v)

6.2.1.2 qRT-PCR

Total RNA was extracted and first strand cDNA synthesised as per sections 2.9.1 and 2.9.2. qRT-PCR was then performed as per section 2.9.4. The primers detailed in Table 2.11 and 2.12 were utilised depending on the species of cells. Primers for *POU5F1, LIN28, PRDM1, IFITM3* and *C-KIT* were also used in putative bovine OSC experiments (detailed in Table 2.4). In putative bovine OSC experiments, 150ng of total RNA was used for cDNA synthesis, in putative human OSC experiments, 150 – 300ng was used and in fetal bovine somatic cell experiments, 200ng was utilised.

6.2.1.3 Immunocytochemistry

To analyse the protein expression of genes of interest, ICC experiments were performed, as detailed in section 2.7. Cells were serum- and LIF-starved overnight as per section 6.2.1.1. The following day, cells were allocated to one of two treatment groups: (1) BMP4 and RA or (2) vehicles only (concentrations as per Table 6.1). After

24 hours, cells were washed and fixed in 4% NBF for 10 mins. NBF was used as it was the required fixative for the antibody against SYCP3. Normal chicken serum was used as a blocking agent. The primary and secondary antibodies utilised are detailed in Table 6.2.

Table 6.2. Antibodies used for immunocytochemical analysis of BMP4 plus RA or vehicle-treated cells.

Primary Antibody	Product Number	Species Raised	Dilution	Source
ID1	sc-488	Rabbit Polyclonal	1:100	Santa Cruz
MSX2	sc-15396	Rabbit Polyclonal	1:100	Santa Cruz
SYCP3	ab15093	Rabbit Polyclonal	1:10000	Abcam
Secondary Antibody		**Product Number**	**Dilution**	**Source**
Chicken anti-rabbit		sc-2963	1:200	Santa Cruz

Green tyramide signal amplification and either PI (putative bovine OSC experiments) or DAPI (putative human OSC and fetal bovine somatic cell experiments) was used as a nuclear counterstain. The primary antibody was omitted for the purposes of negative controls. Slides were analysed and images were acquired as per section 2.8.2.

6.2.2 *DAZL* transfection experiments

To investigate whether an increase in endogenous *DAZL* would enhance the expression of meiotic genes, putative bovine and human OSCs were transfected with a DAZL-containing plasmid. These experiments were performed in collaboration with Dr. Roseanne Rosario (post-doctoral scientist in Prof. Anderson's group).

6.2.2.1 Liposomal-mediated transfection

Putative OSCs were cultured in 24-, 12- or 6-well plates in antibiotic-free OSC culture medium for at least 24 hours prior to transfection and were not transfected until they were approximately 70% confluent. On the day of transfection, lipid/DNA complexes were prepared in a laminar flow hood as per the manufacturer's instructions, with a final plasmid concentration of 1µg/ml (Table 6.3). The lipid transfection reagent encapsulates the DNA to form a liposome, enabling uptake through the hydrophobic cell membrane (Fig. 6.3).

Table 6.3. Lipid/DNA complex constituents used for *DAZL* transfection of putative OSCs. * The GFP plasmid was not used in all experiments. See text for further details.

Reagent	Amount (µl)			Source
	Culture plate			
	24 well	12 well	6 well	
Opti-MEM® (1X) + GlutaMAX™ (serum-free medium)	50	100	250	Life Technologies
TransIT®-LT1 lipid transfection reagent	1.5	3	7.5	Mirus
DNA plasmid (500ng/µl) *OR* Control plasmid (500ng/µl)	1	2	5	OriGene
*GFP plasmid (500ng/µl)**	*1*	*N/A*	*5*	*Clontech Laboratories, Inc.*

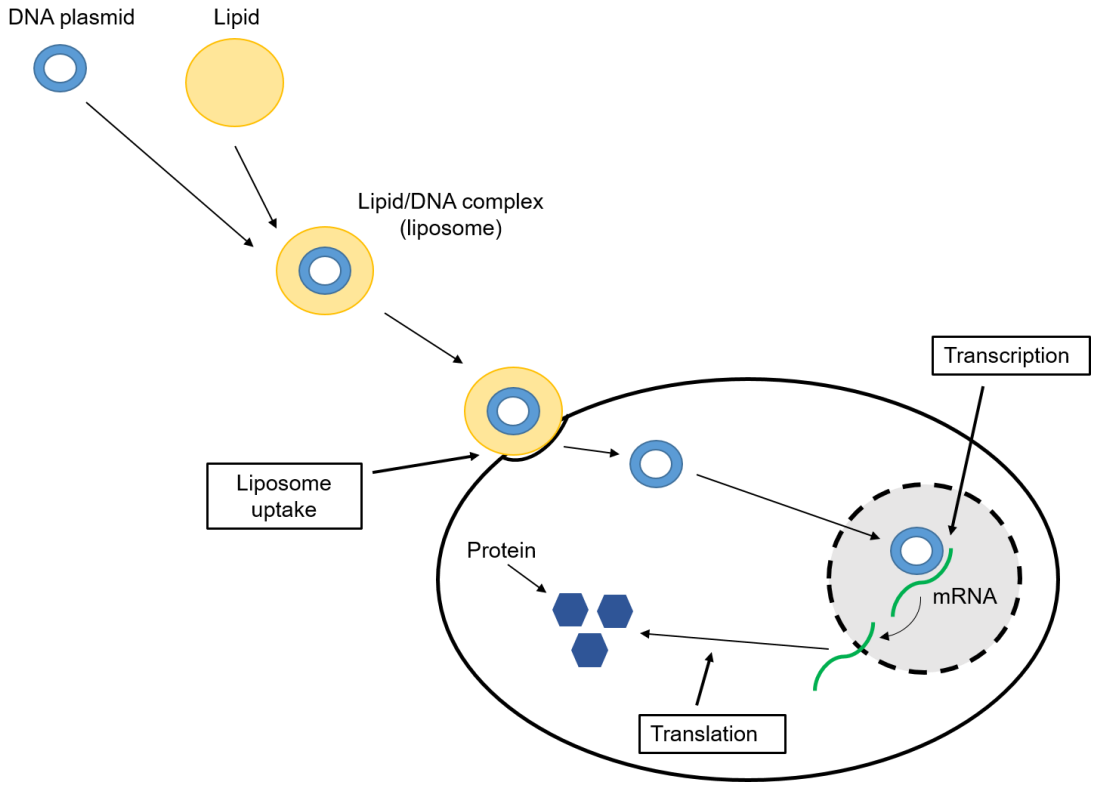


Figure 6.3. Schematic of liposomal-mediated transfection. Lipid/DNA complexes are formed during incubation of the DNA plasmid and lipid transfection reagent. These liposomes can enter the cell via the hydrophobic membrane. The DNA is transcribed to mRNA in the nucleus and translated by cytoplasmic ribosomes to produce the desired protein.

Cells were transfected with either a DAZL plasmid (TrueORF cDNA clone pCMV6-entry plasmid containing human DAZL transcript variant 2, Myc-DDK-tagged; accession number: NM_001351) or an empty plasmid (pCMV6-entry plasmid, Myc-DDK-tagged with no coding sequence downstream of the CMV promoter), which acted as a control to assess whether transfection itself affected the cells' viability.

The lipid/DNA complexes were incubated at room temperature for 20 mins before being added to fresh antibiotic-free OSC medium (0.5ml for 24 well plates, 1ml for 12 well plates and 5ml for 6 well plates). The medium of the cells was then replaced with this plasmid-containing medium. Cells were cultured at 37ºC / 5% CO_2 for 24, 48 or

72 hours before being collected for either qRT-PCR (bovine cells) or Western blotting (bovine and human cells).

Subsequent experiments involving enrichment for transfected cells by flow cytometry required cells to be co-transfected with a GFP-containing plasmid (pEGFP-C1 plasmid with CMV promoter; Clontech Laboratories, Inc.). As with the DAZL and control plasmids, a final concentration of 1µg/ml was used.

6.2.2.2 Treatment of transfected cells

To determine if treatment with a combination of *DAZL* transfection and BMP4 plus RA treatment would affect downstream expression of genes of interest, a 5 day experiment was devised (Fig. 6.4). Putative human OSCs (Patient 1, Population B, P10) were passaged on Day 1 so that they would be approximately 70% confluent the following day. Transfection with DAZL or control plasmid was performed on Day 2. On Day 3, the antibiotic-free OSC culture medium was changed to OSC culture medium without antibiotics, serum or LIF. On Day 4, cells were allocated to one of two treatment groups: (1) BMP4 and RA or (2) vehicles only (concentrations as per Table 6.1). On Day 5, cells were collected for analysis by trypsinisation, centrifugation at 800 x g for 5 mins and resuspension in 350µl RLT buffer containing 1% (v/v) β-ME.

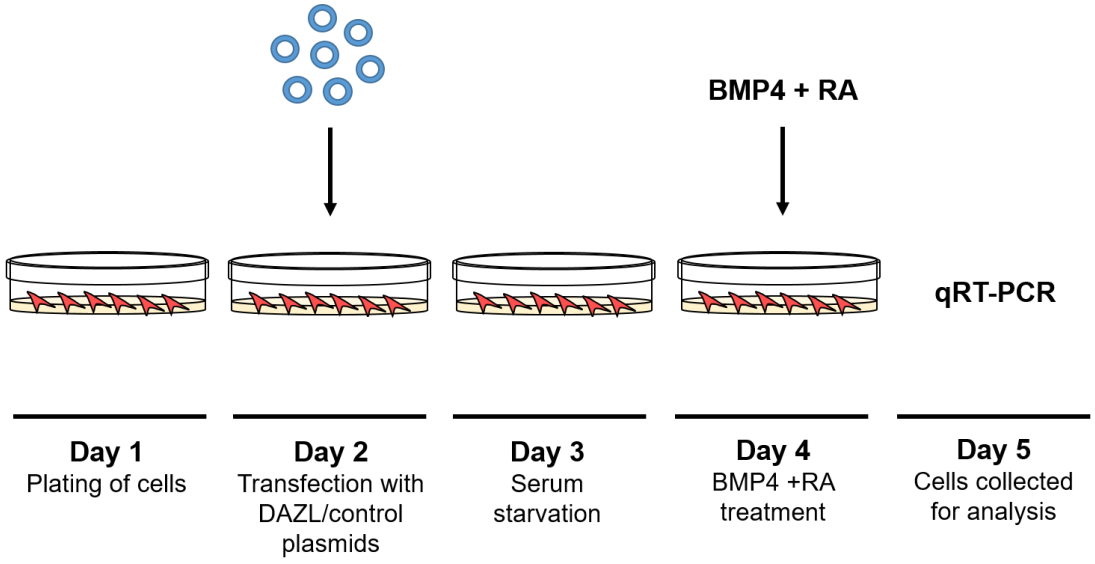


Figure 6.4. Methodology for combined transfection and BMP4 plus RA treatment experiments. After passaging on the first day to achieve the correct confluence, cells were transfected the following day with either DAZL plasmids or empty (control) plasmids. Forty-eight hours later, and following serum- and LIF-starvation, cells were treated with BMP4 and RA for 24 hours. Cells were then collected for gene expression analysis.

In subsequent experiments (Fig. 6.5), enrichment for transfected cells was performed by co-transfecting the cells with a GFP plasmid. The protocol above was repeated with both bovine cells (Cell Line 4, Population B, P17 and P19) and human cells (Patient 1, Population B, P12 and P15), but GFP plasmid was added on the day of transfection and cells were collected in 0.5ml DPBS. They were then subjected to FACS (using the BD FACSAriaTM II cytometer at QMRI, University of Edinburgh), with GFP expression being used to enrich for DAZL or control transfected cells. Staff at the Facility (Shonna Johnston and Will Ramsay) performed the analysis and cell sorting in discussion with myself. Transfected cell samples were compared to cells which had not been exposed to GFP plasmids and gates were set up using a fluorescence wavelength of 525nm in order to select GFP-positive cells. The cells were collected into 1ml of PBS containing 10% (w/v) BSA and 0.5M EDTA in 1.5ml microfuge tubes and transported back to the laboratory for analysis.

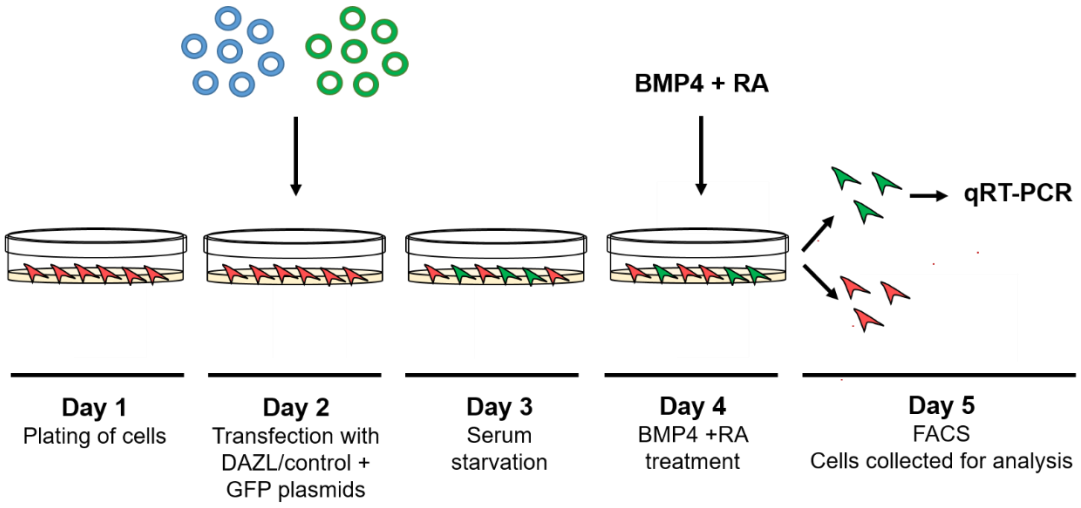


Figure 6.5. Modified methodology for combined transfection and BMP4 plus RA treatment experiments, allowing enrichment of transfected cells. On Day 2, cells were transfected with DAZL plasmids or control plasmids plus GFP plasmids. Following BMP4 and RA treatment, cells were subjected to FACS and transfected cells were selected on the basis of GFP expression. This enriched cell population was then analysed for gene expression.

6.2.2.3 qRT-PCR

For experiments where FACS enrichment was not performed, total RNA was extracted and first strand cDNA was synthesised as per sections 2.9.1 and 2.9.2. If possible, 500ng of RNA was used for cDNA synthesis. For experiments where enrichment was performed, total RNA was extracted using the ARCTURUS® PicoPure® RNA Isolation kit and cDNA was synthesised using the Superscript® III First-Strand Synthesis System as per section 4.2.1.2.1. Total RNA concentrations were extremely low in these experiments, therefore all RNA was used for cDNA synthesis. qRT-PCR was subsequently performed in all experiments as per section 2.9.4. The primers detailed in Table 2.11 and 2.12 were utilised.

6.2.2.4 Western blotting

DAZL-transfected, non-enriched putative bovine and human cells were analysed for protein expression after 24, 48 and 72 hours of transfection by Western blotting as per

section 2.10. Bovine Cell Line 4, Population B, P19 cells and human Patient 8 Population A, P13 cells were analysed. Thirty micrograms of protein was loaded in each experiment. Cells were compared to either control plasmid-transfected cells (bovine) or non-transfected cells (human) of the same cell lines and passages. Tables 6.4 and 6.5 detail the primary and secondary antibodies used.

Table 6.4. Primary antibodies used to analyse DAZL expression by Western blotting.

Primary Antibody	Product Number	Species Raised	Dilution	Source
DAZL	#8042	Rabbit Polyclonal	1:1000	Cell Signaling Technology
α-tubulin	T-6074	Mouse Monoclonal	1:1000	Sigma-Aldrich

Table 6.5. Secondary antibodies used for Western blotting.

Secondary Antibody	Conjugate	Product Number	Dilution	Source
Donkey anti-rabbit	Alexa Fluor® 680	A10043	1:10000	Thermo Scientific
Donkey anti-Mouse	IRDye® 800CW	925-32212	1:10000	Li-cor

Proteins of interest were detected using secondary antibodies visible in the 680nm channel (i.e. red) of the Li-cor Classic infrared imaging system. Alpha-tubulin (α-tubulin) was used as a loading control and was detected with a secondary antibody visible in the 800nm channel (i.e. green). In the bovine experiment, human embryonic

kidney 293 (HEK-293) cells transfected with the same DAZL plasmid (kindly donated by Dr. Roseanne Rosario) were used as a positive control.

6.2.3 Statistical Analyses

Gene expression in the qRT-PCR experiments was calculated and analysed as per section 2.9.4.1. In brief, normalised gene expression was calculated using the $\Delta\Delta C_q$ method (Livak and Schmittgen, 2001), with comparison to the expression of the references genes *β-actin* (bovine samples) and *RPL32* (human samples). Calculated $2^{-\Delta\Delta C_q}$ values were logarithmically transformed and checked for normalcy. Normally distributed data was analysed with the parametric one-way analysis of variance (ANOVA) with a post-hoc Tukey test. Data that was not normally distributed was analysed with the non-parametric Kruskal-Wallis test with post-hoc Dunn's test. A p level of < 0.05 was considered significant.

To analyse the ICC experiments, ImageJ software (National Institutes of Health) was used to perform staining intensity comparisons. The software was used to select areas of cytoplasmic or nuclear staining (Fig. 6.6). The red, green or blue colour was then converted by the software to grayscale using the following formula: *gray = 0.299red + 0.587green + 0.114blue*. The mean gray value was subsequently calculated by dividing the sum of the grayscale values of the pixels in the selected areas by the number of pixels. As the same laser settings were used for every image, the nuclear staining intensity should be similar, therefore in order to account for differing number of cells in each image, the cytoplasmic (i.e. green) staining was compared to the nuclear (i.e. red or blue) staining of the same image by dividing the cytoplasmic mean gray value by the nuclear mean gray value. The resultant non-treated cell values were compared to treated cell values for each protein: by standardising the values to 1 (i.e. by dividing both values by the non-treated cell value), an estimate of fold change in staining intensity was obtained.

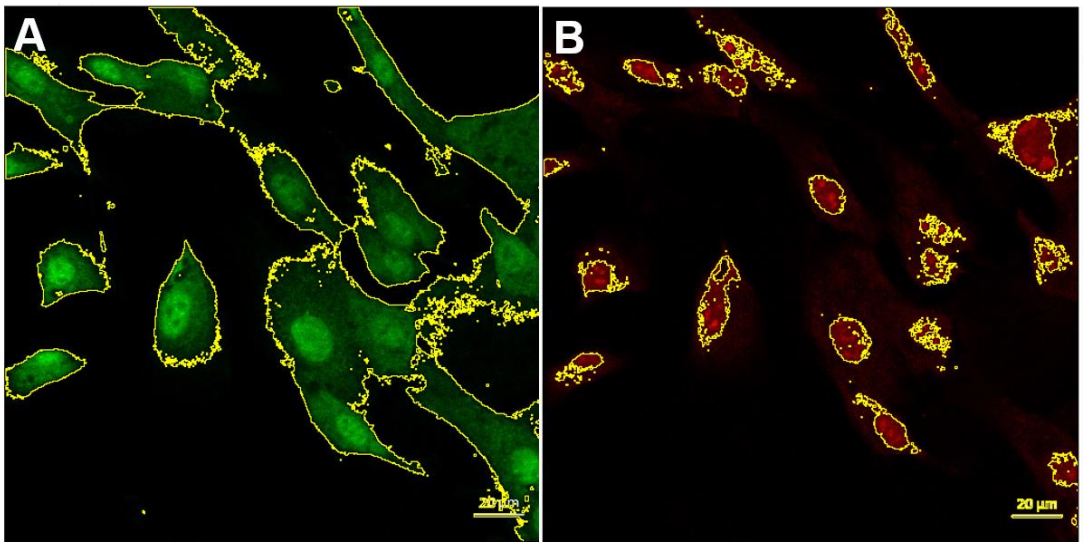


Figure 6.6. Quantification of staining of proteins of interest was performed using ImageJ software. Following acquisition of images of the cells using a confocal microscope, ImageJ software was utilised to select and quantify areas of **(A)** cytoplasmic and **(B)** nuclear staining. Further details on the quantification methods are described in the text. The images are representative of putative bovine OSCs treated with BMP4 and RA and stained for ID1.

6.3 Results

6.3.1 BMP4 and/or RA treatment experiments

6.3.1.1 Putative bovine OSC experiments

Twenty-four hour treatment of putative bovine OSCs with BMP4 alone or combined with RA resulted in significant upregulation of gene expression of all the analysed genes: *ID1*, *MSX1*, *MSX2* and *SYCP3* (Fig. 6.7; n = 6 or 7 per treatment). *ID1* and *MSX2* mRNA expression was significantly upregulated with BMP4 treatment alone ($p < 0.0001$ and $p < 0.01$ respectively), but dual treatment with both BMP4 and RA augmented this effect, with a significant increase in expression above that of BMP4 treatment only observed ($p < 0.05$ for *ID1* and < 0.001 for *MSX2*). *MSX1* expression was not increased with BMP4 alone, but did increase significantly with combined BMP4 and RA treatment ($p < 0.01$). Furthermore, combined treatment resulted in significantly increased expression above that of BMP4 only treated cells ($p < 0.0001$), but not RA only treated cells. Both BMP4 alone and combined treatment with BMP4 and RA significantly increased the expression of *SYCP3* (both $p < 0.0001$). *STRA8* was not reliably detected. Bovine primers for *RARβ* were designed, however, the mRNA sequence on the NCBI Nucleotide database is a predicted sequence only and the primers were unable to detect expression in a positive control (bovine fetal ovary). The primers were therefore not validated for use. No OLCs were observed during the culture period.

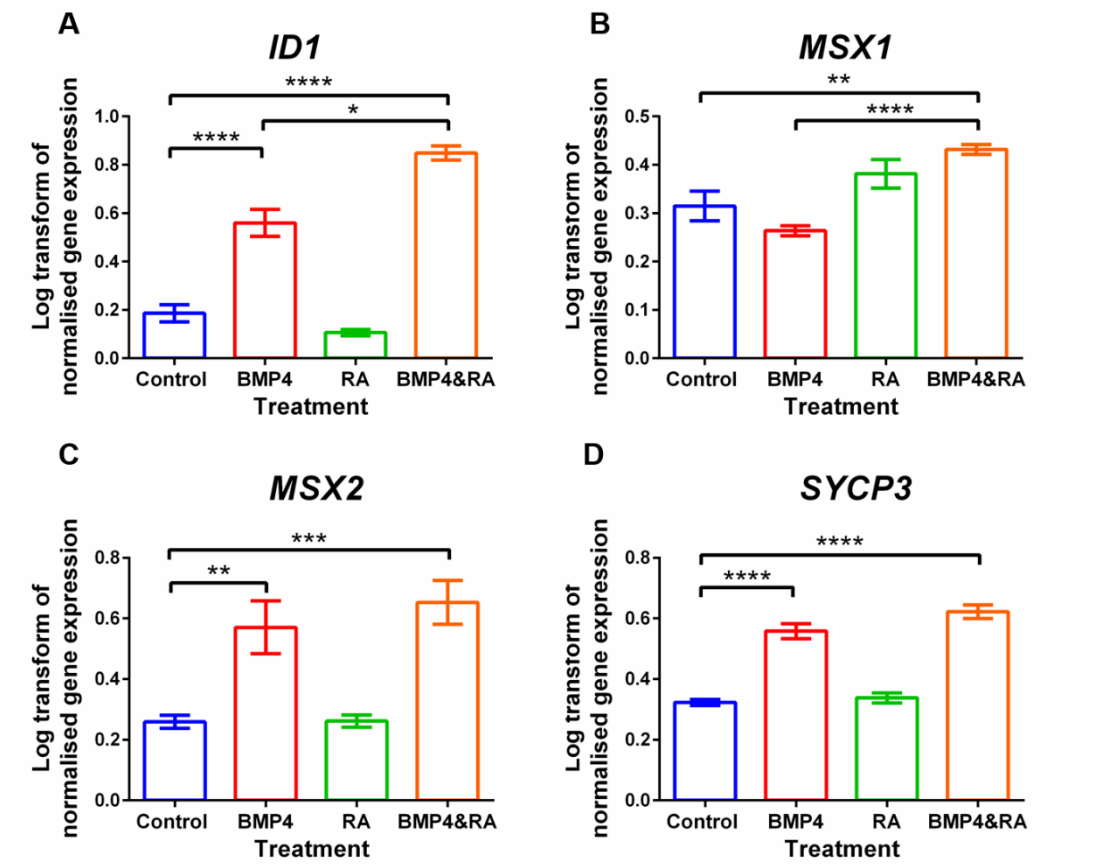


Figure 6.7. Expression of BMP4- and RA-response genes in putative bovine OSCs that had been treated with BMP4 and/or RA for 24 hours was analysed by qRT-PCR. **(A)**, **(C)** and **(D)** *ID1*, *MSX2* and *SYCP3* expression increased significantly with BMP4 only treatment and a synergistic effect was seen when combined treatment of BMP4 and RA was used in the case of *ID1* and *MSX2*. **(B)** In contrast, *MSX1* expression was significantly upregulated only in the presence of RA, with expression in response to combined treatment significantly higher than both control and BMP4 treated cells. (*$p < 0.05$, **$p < 0.01$, *** $p < 0.001$, ****$p < 0.0001$; mean ± S.E.M.; n = 6 or 7 replicates per treatment group performed during 2 separate experiments).

The expression of stem cell (*LIN28*, *POU5F1*) and germ cell (*IFITM3*, *C-KIT*) markers did not significantly change with any treatment, with the exception of *PRDM1*, where a non-significant trend in increased expression was observed with BMP4 treatment

only but combined treatment with both BMP4 and RA significantly upregulated its expression ($p < 0.01$); Fig. 6.8).

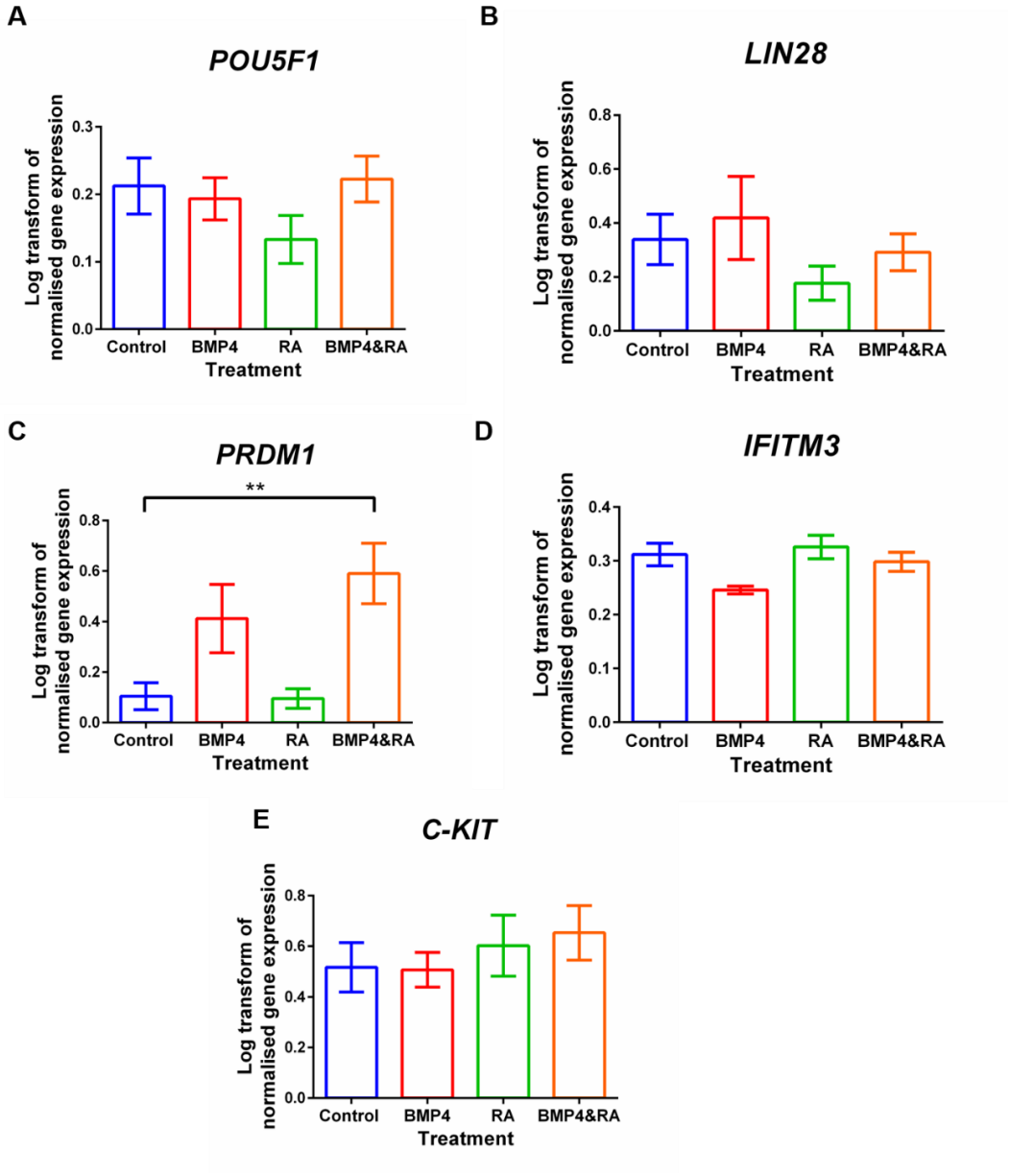
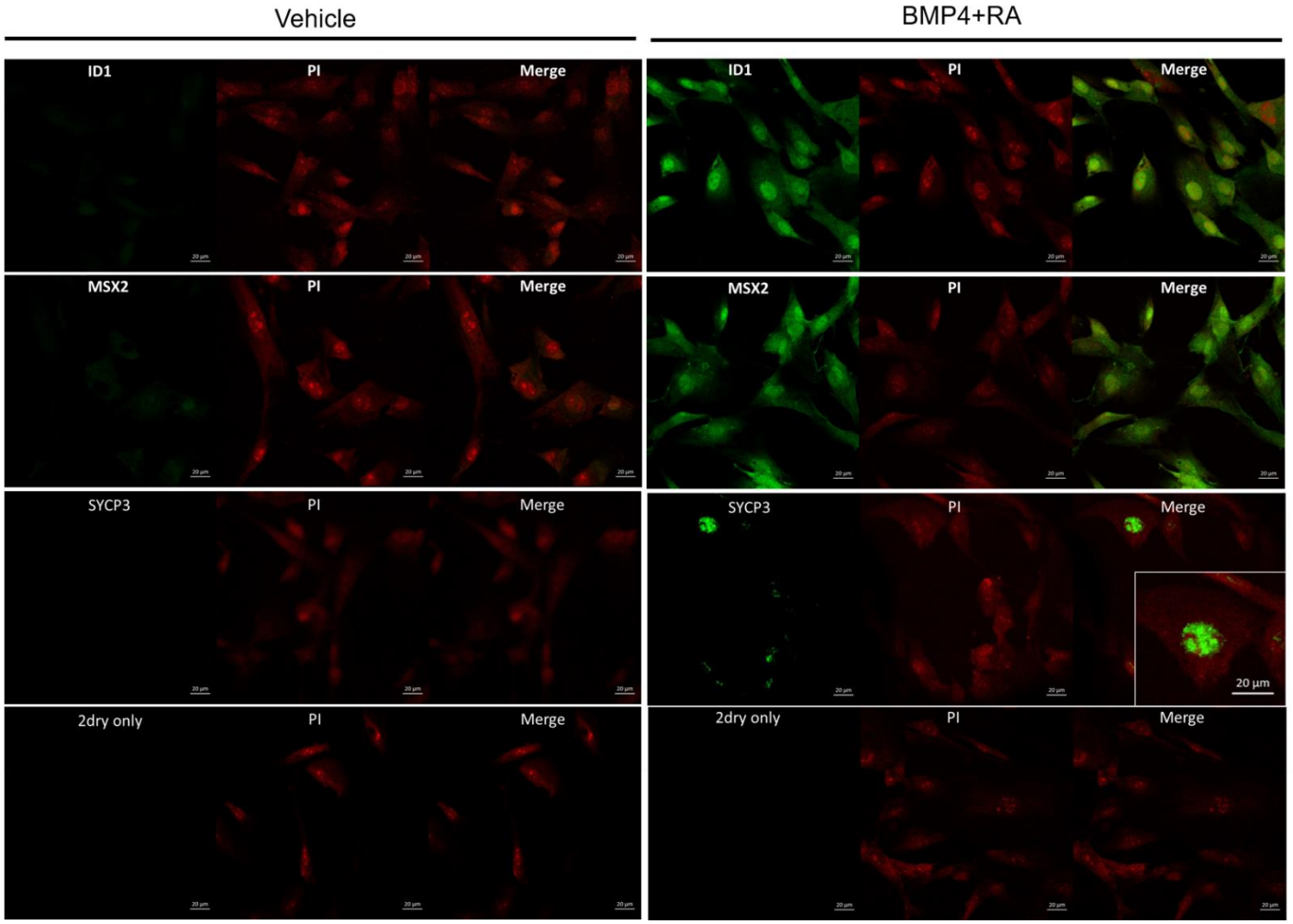


Figure 6.8. Expression of pluripotency and germline markers putative in bovine OSCs that had been treated with BMP4 and/or RA for 24 hours. (A), **(B)**, **(D)** and **(E)** *POU5F1*, *LIN28*, *IFITM3* and *C-KIT* expression was not affected significantly by treatment. **(C)** In contrast, *PRDM1* expression was significantly upregulated by exposure to combined BMP4 plus RA treatment. (**p < 0.01; mean ± S.E.M.; n = 6 or 7 replicates per treatment group performed during 2 separate experiments).

Protein expression of BMP4- and RA-response genes was also affected by treatment, as demonstrated by ICC. Due to cell availability only two treatment groups could be allocated: given that combined BMP4 and RA treatment led to the greatest increase in mRNA expression, cells were exposed to either vehicle controls or BMP4 with RA. ID1 and MSX2 were located in both the cytoplasm and the nucleus whereas SYCP3 was located exclusively in the nucleus. Although the ID proteins inhibit DNA binding of basic helix-loop-helix proteins, ID1 can be located in the cytoplasm as well as the nucleus (Coppe *et al.*, 2003, Nishiyama *et al.*, 2007). MSX2 can similarly be found in both cellular compartments (Lanigan *et al.*, 2010, Gremel *et al.*, 2011).

Qualitative analysis of cultured cells treated with combined BMP4 plus RA treatment suggested an increase in protein expression of ID1, MSX2 and SYCP3 compared to vehicle-treated cells (Fig. 6.9). This also allowed an estimate of the fold change in expression: the staining intensity of ID1 in BMP4 and RA-treated cells was 7 times that of vehicle-treated cells, whilst the intensity of MSX2 was 4 times higher in BMP4 and RA-treated cells. Quantitative analysis of SYCP3 staining established that 50% of treated cells were SYCP3-positive, compared to 0% of vehicle-treated cells.

Figure 6.9. Immunocytochemistry of putative bovine OSCs treated with either vehicle or BMP4 plus RA. BMP4 plus RA-treated cells demonstrated increased expression of ID1, MSX2 and SYCP3 compared with vehicle controls. Propidium iodide (PI) was used as a nuclear counterstain. An enlarged image of a SYCP3-positive cell is shown. The primary antibody was omitted for the purposes of a negative control. Scale bars = 20μm.


Vehicle
BMP4+RA
ID1
PI
Merge
MSX2
SYCP3
2dry only
20 µm

6.3.1.2 Putative human OSC experiments

ID1 was the only gene analysed in the bovine experiments that was significantly affected by treatment in the human experiments (Fig. 6.10; n = 6 - 8 per treatment). Gene expression of *ID1* was again significantly upregulated by BMP4 treatment alone or combined treatment with RA, although the addition of RA did not result in any further significant upregulation, in contrast to the bovine studies. The cells were also shown to be RA-responsive, with *RARβ* expression significantly upregulated in response to RA treatment alone or in combined treatment with BMP4. BMP4 only treatment did not increase the gene's expression. *MSX1* expression was only detected in three experiments: although there was a trend for increased expression with RA treatment, it was not significant. *MSX2* expression was undetectable in all experiments. *SYCP3* expression showed a trend for upregulation with BMP4 and RA treatment, however, this did not reach significance. As in the bovine experiments, no OLCs were observed during the culture period.

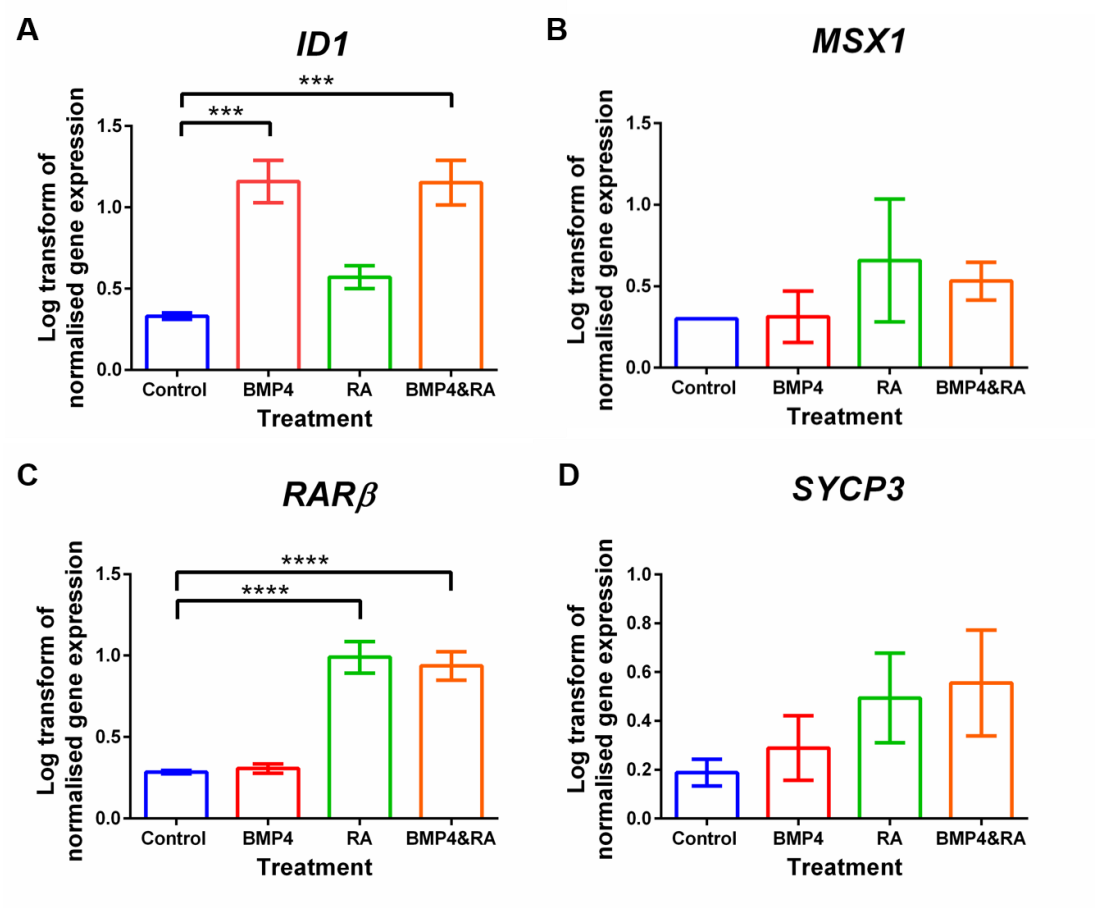
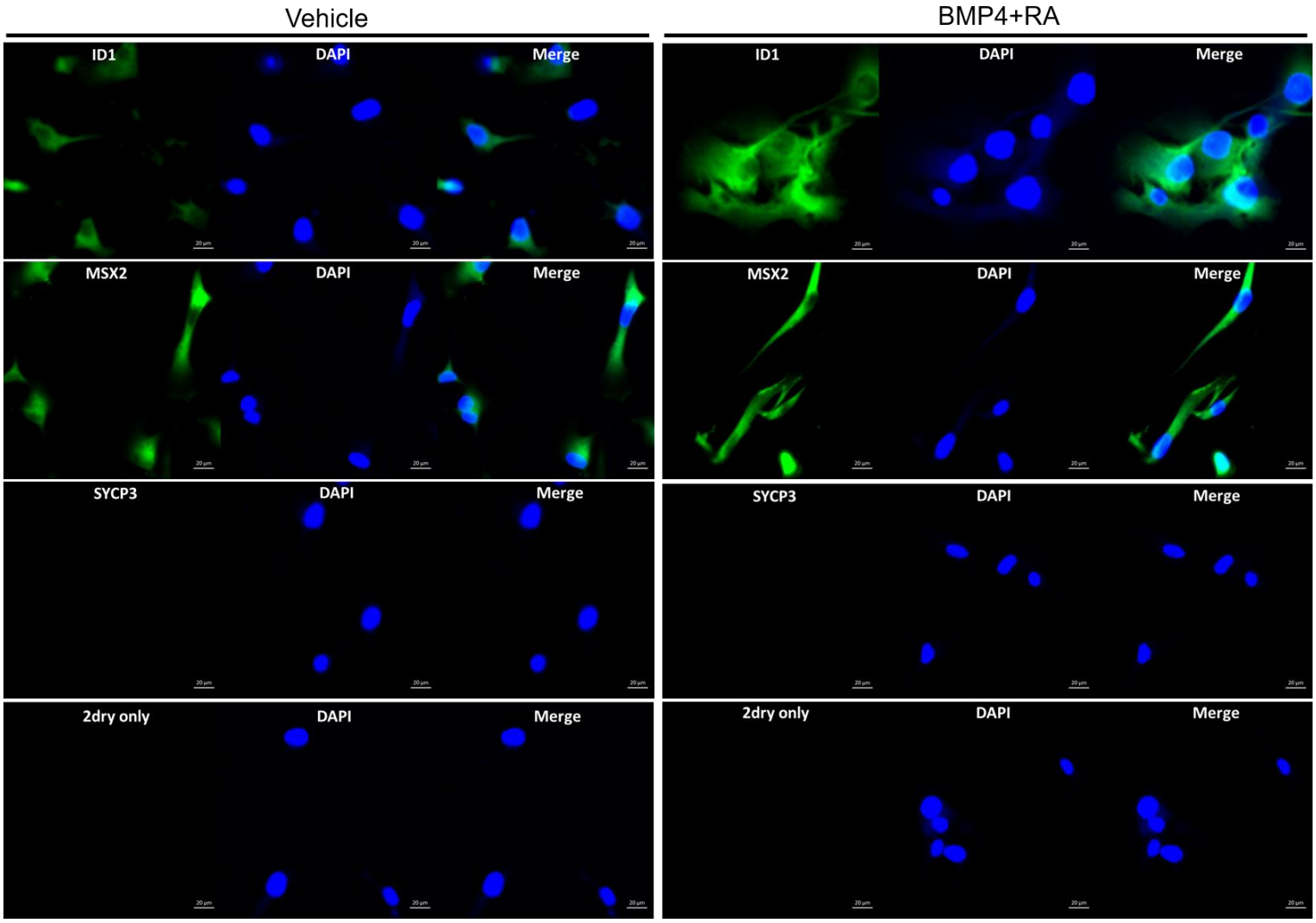


Figure 6.10. Expression of BMP4- and RA-response genes in putative human OSCs that had been treated with BMP4 and/or RA for 24 hours. (A) *ID1* expression increased significantly with BMP4 only treatment and combined BMP4 plus RA treatment. **(B)** *MSX1* was only detectable in three experiments and showed no significant changes in expression. **(C)** The cells demonstrated RA-responsiveness, with significant upregulation of *RARβ* if RA was included in treatment. **(D)** *SYCP3* expression exhibited a non-significant trend for increased expression with RA only and BMP4 plus RA treatment (*** $p < 0.001$, ****$p < 0.0001$; mean ± S.E.M.; n = 6 - 8 replicates per treatment group, performed during 4 separate experiments, with exception of *MSX1*, where n = 3 replicates performed during 3 separate experiments).

Contrary to the bovine ICC results, increased protein expression of ID1 or MSX2 was not suggested in BMP4 and RA-treated cells compared to vehicle-treated cells, either qualitatively or quantitatively (Fig. 6.11). ID1 staining intensity was calculated to be 1.1 times higher in the BMP4 and RA-treated cells, while MSX2 intensity was 1.2 times higher, indicating no differences. No cells in either treatment group demonstrated SYCP3 expression.

Figure 6.11. Immunocytochemistry of putative human OSCs treated with either vehicle or BMP4 plus RA. No change in ID1 or MSX2 protein expression was observed with treatment. SYCP3 expression was not detected in any cells. DAPI was used as a nuclear counterstain. The primary antibody was omitted for the purposes of a negative control. Scale bars = 20μm.


Vehicle
BMP4+RA
ID1
DAPI
Merge
MSX2
SYCP3
2dry only

6.3.1.3 Fetal bovine somatic cell experiments

To investigate whether other types of ovarian cells were similarly BMP4 and/or RA responsive, fetal bovine somatic cells were treated and analysed for mRNA and protein expression. Although the cells appeared to be BMP4-responsive, with trends for upregulation of *ID1*, *MSX1* and *MSX2* with BMP4 or combined BMP4 and RA treatment, these trends did not reach significance (Fig. 6.12; n = 3). Neither *SYCP3* nor *STRA8* demonstrated any significant change in expression with treatment.

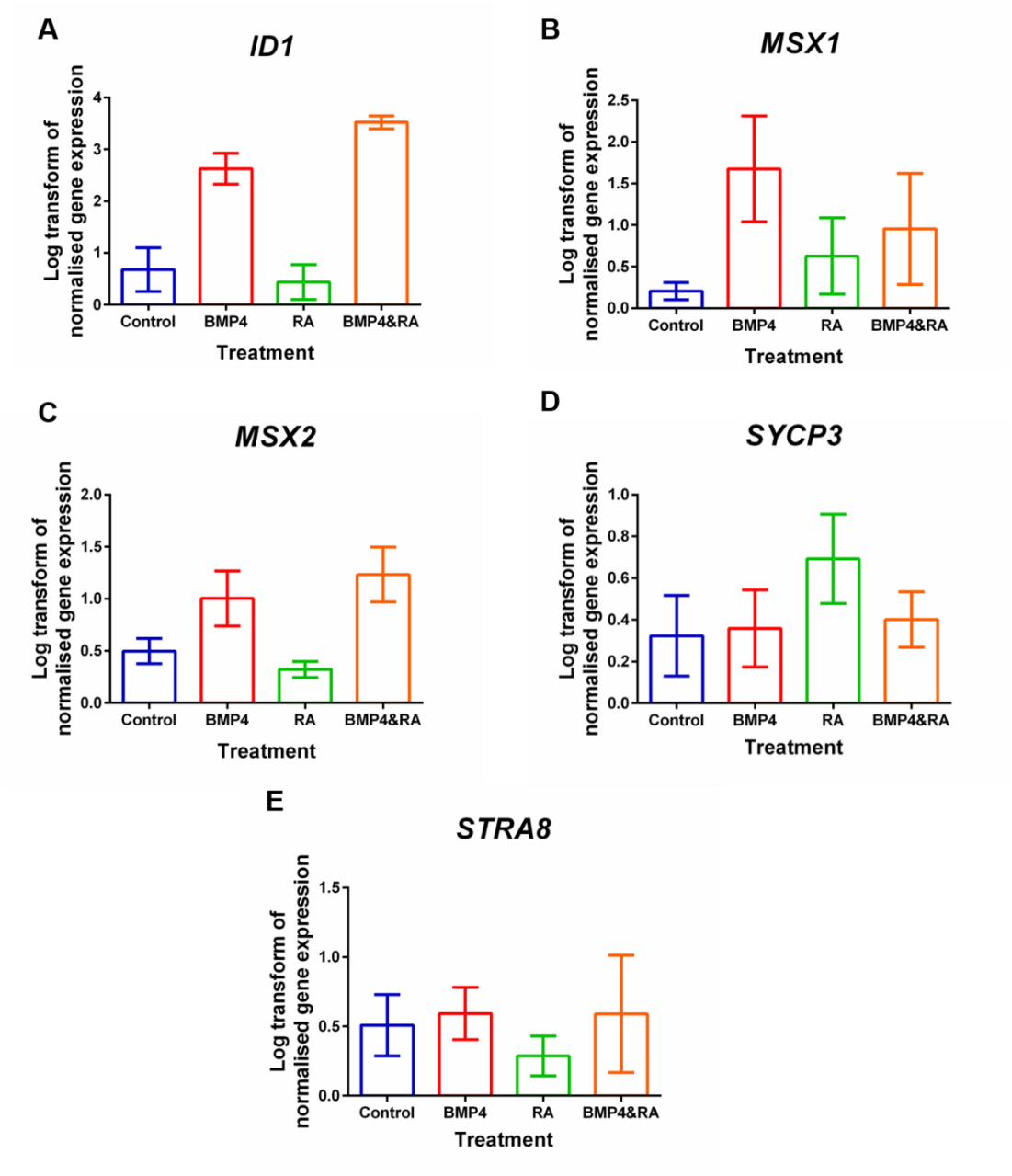
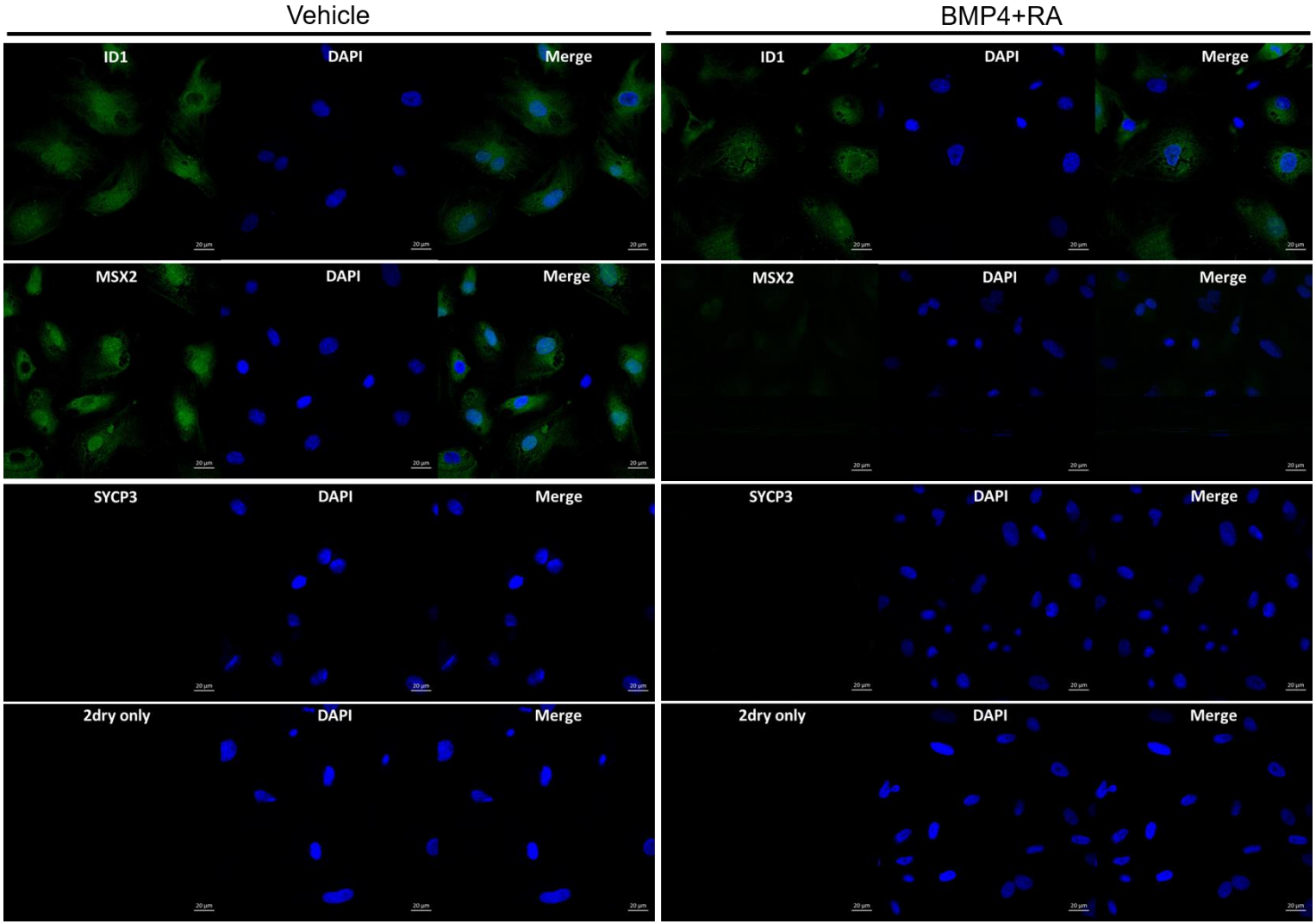


Figure 6.12. Expression of BMP4- and RA-response genes in fetal bovine somatic cells that had been treated with BMP4 and/or RA for 24 hours. (A), **(B)** and **(C)** The cells demonstrated BMP4-responsiveness, with non-significant upregulation of *ID1*, *MSX1* and *MSX2* with BMP4 only treatment or and/or combined BMP4 plus RA treatment. **(D)** and **(E)** Expression of the meiotic entry genes, *SYCP3* and *STRA8*, was not significantly altered with treatment. (Mean ± S.E.M.; n = 3 replicates per treatment group performed during 1 experiment).

Immunocytochemical analysis demonstrated similar results to the human experiments, with no qualitative or quantitative difference in protein expression of ID1 or MSX2 between BMP4 and RA-treated cells and vehicle-treated cells (Fig. 6.13). ID1 staining intensity was calculated to be the same in both treatment groups, while MSX2 intensity was actually 1.7 times higher in the vehicle-treated cells. SYCP3 expression was not detected in any cells in either treatment group.

Figure 6.13. Immunocytochemistry of fetal bovine somatic cells treated with either vehicle or BMP4 plus RA. No increased expression of ID1 or MSX2 protein was observed with treatment. SYCP3 expression was not detected in any cells. DAPI was used as a nuclear counterstain. The primary antibody was omitted for the purposes of a negative control. Scale bars = 20μm.


Vehicle
BMP4+RA
ID1
DAPI
Merge
MSX2
SYCP3
2dry only

6.3.2 *DAZL* transfection experiments

Given the key role of DAZL in meiosis (Lin *et al.*, 2008) and its ability to induce hESCs and iPSCs to form PGCLCs (Medrano *et al.*, 2012), an experiment was designed to investigate whether overexpression of DAZL in putative OSCs would result in increased expression of meiosis-related genes. A liposomal-mediated transfection technique was utilised to introduce the DAZL plasmid into the cells.

6.3.2.1 Non-enriched experiments

A preliminary experiment was performed using putative bovine OSCs (Cell Line 4, P19; n = 1 per treatment group) to determine whether (a) the cells could be transfected to upregulate the expression of *DAZL* and (b) if the transfection was effective, how long it took for increased *DAZL* expression to be detected. A time course experiment demonstrated that the transfection was successful after 24 hours of transfection, but that there was a further increase in *DAZL* expression after 48 hours (Fig. 6.14). There was little difference between 48 and 72 hours. Statistical analysis for significance was not possible as n = 1 per treatment group.

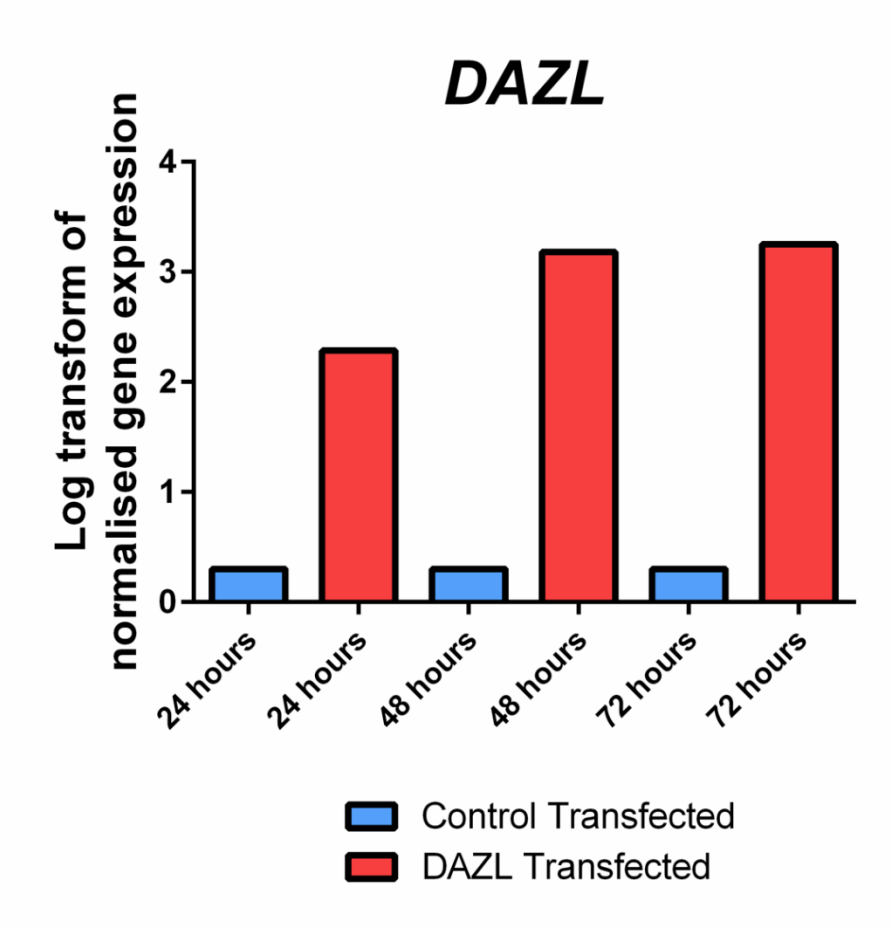


Figure 6.14. Preliminary transfection experiments utilising putative bovine OSCs demonstrated that transfection with a DAZL overexpression plasmid resulted in upregulation of *DAZL* expression, with a plateau of expression at 48 – 72 hours. (Mean; n = 1 replicate per treatment group performed during 1 experiment).

The same cells were then analysed for *ID1*, *MSX1*, *MSX2* and *SYCP3* expression (Fig. 6.15). Again, statistical analysis for significant changes in gene expression was not possible, although, there appeared to be a slight trend for upregulation of expression with time for *ID1*, *MSX1* and *MSX2* in DAZL transfected cells. However, DAZL transfection was not associated with clear effects on the expression of these genes compared with the control plasmid-transfected cells.

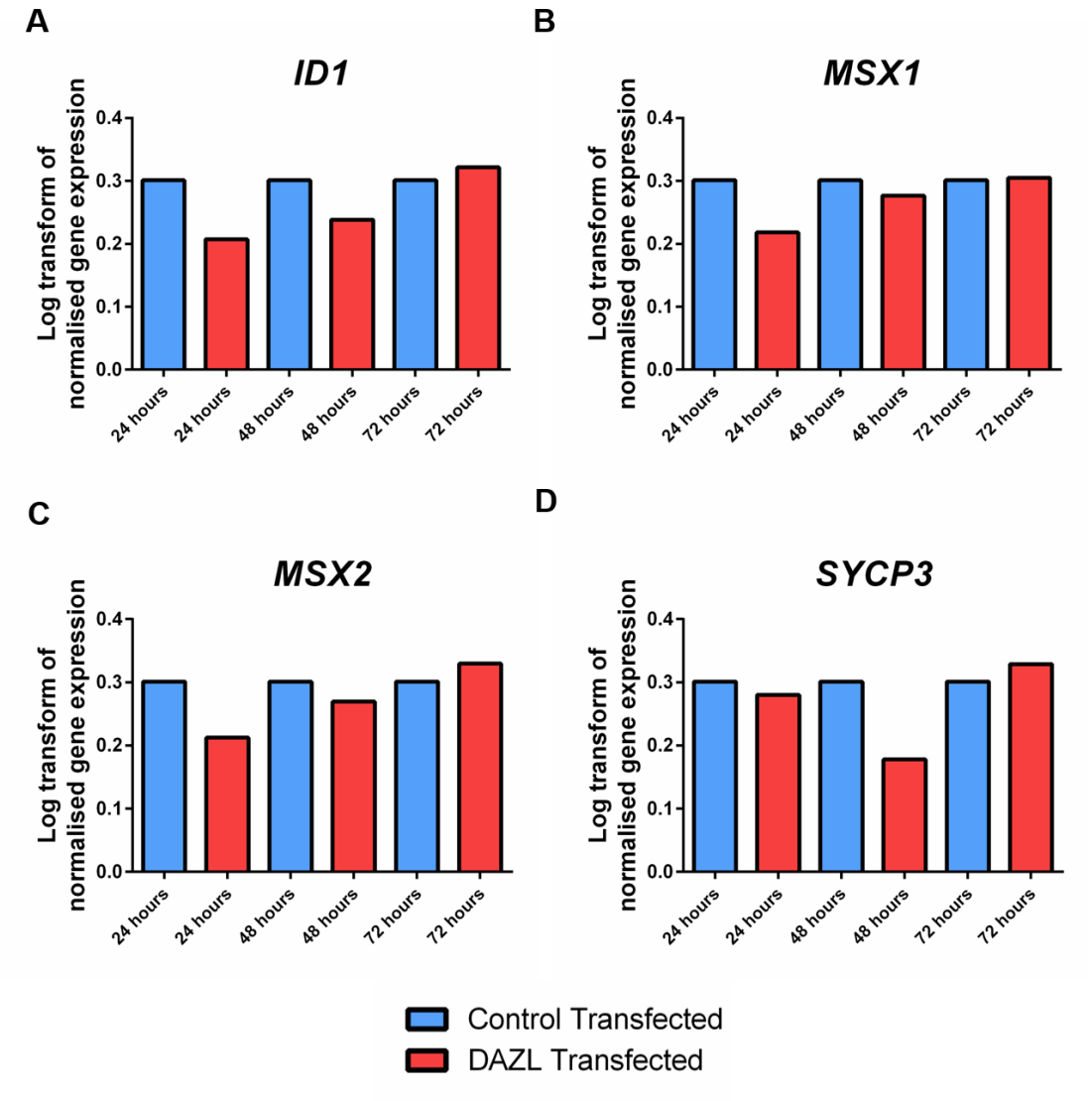


Figure 6.15. Analysis of downstream genes demonstrated that DAZL transfection alone did not significantly affect gene expression at any of the chosen time points. (Mean; n = 1 replicate per treatment group performed during 1 experiment)

A DAZL transfection and BMP4 plus RA treatment experiment was subsequently performed to analyse whether a combination of upregulation of *DAZL* expression and treatment would affect downstream gene expression. A period of 48 hours between transfection and treatment was chosen as the time course experiment above indicated that transcription levels were high at this time point. This experiment, utilising

putative human OSCs, demonstrated that human cells could also be transfected to upregulate expression of *DAZL*, although this did not reach significance, possibly due to the low n numbers (Fig. 6.16; n = 3 per treatment).

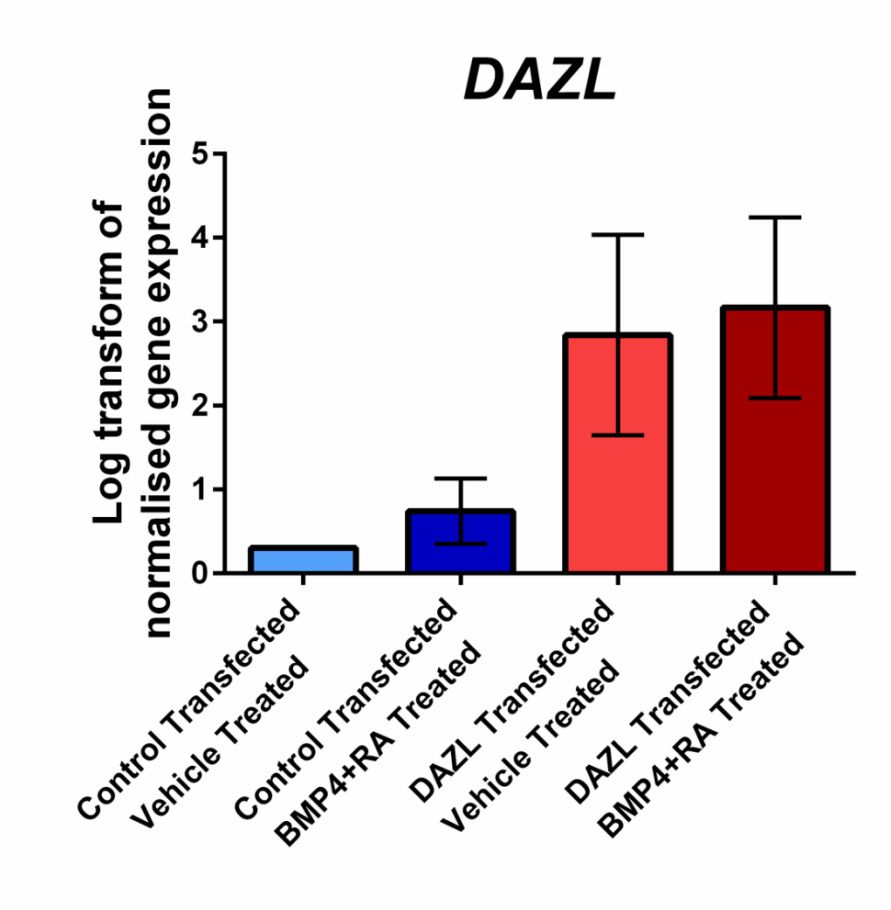


Figure 6.16. DAZL transfection of putative human OSCs resulted in upregulation of *DAZL* expression at non-significant levels. (Mean ± S.E.M.; n = 3 replicates per treatment group performed during 3 separate experiments).

Analysis of the downstream genes revealed that, as expected, *ID1* and *RARβ* showed evidence for upregulation with combined BMP4 and RA treatment; however, *DAZL* transfection did not augment this effect (Fig. 6.17). *MSX1* was only detectable in one experiment, therefore, although it appears *DAZL* overexpression caused increased *MSX1* expression in vehicle-treated cells compared to control transfected and vehicle-treated cells, it is not possible to interpret the results reliably. As seen in the human BMP4 and/or RA treatment experiments described above, *MSX2* was not detectable. The combination of *DAZL* transfection and treatment did appear to result in an increase

in *SYCP3* expression compared to control transfected and either BMP4 plus RA-treated or vehicle-treated cells; however, this did not reach significance.

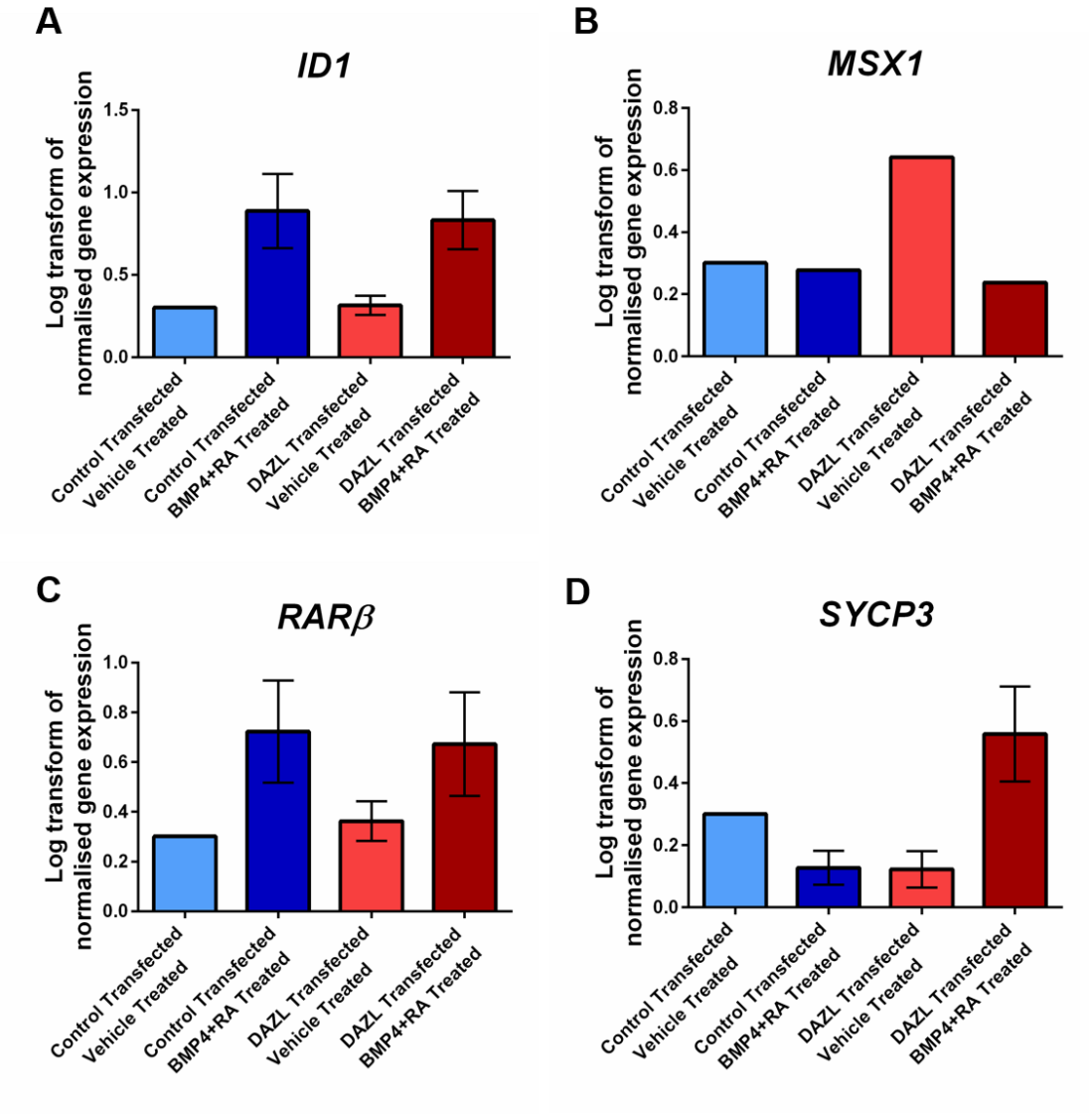


Figure 6.17. Expression of BMP4- and RA-response genes in putative human OSCs that had been transfected with DAZL or control vectors and treated with vehicle or BMP4 plus RA. **(A)** and **(C)** *ID1* and *RARβ* expression exhibited a trend for increased expression with BMP4 plus RA treatment, which was not augmented by *DAZL* overexpression. **(B)** *MSX1* data was difficult to interpret due to only being detectable in 1 experiment. **(D)** *SYCP3* expression was increased in DAZL transfected and BMP4 plus RA treated cells, but the change was not significant. (Mean ± S.E.M.; n = 3 replicates per treatment group performed during 3 separate experiments, with exception of *MSX1*, where n = 1 replicate).

As a result of these experiments, it was postulated that the transfection of the cells may not be efficient and thus any effect on gene expression may be masked by the presence of a majority of non-transfected cells. It was therefore decided to use an enrichment method, in order to select only transfected cells for further gene expression analysis.

6.3.2.2 Enriched experiments

Co-transfection with DAZL or control plasmids and a GFP plasmid was performed in order that transfected cells could be sorted for the presence of GFP by flow cytometry (Fig. 6.18). This utilised the principle that if a cell allowed one plasmid to enter, it would likely take up the other; nevertheless, not all cells containing the DAZL or control plasmid would be GFP-positive and vice versa, therefore this experiment was used for enrichment, not purification, of *DAZL* or control transfected cells.

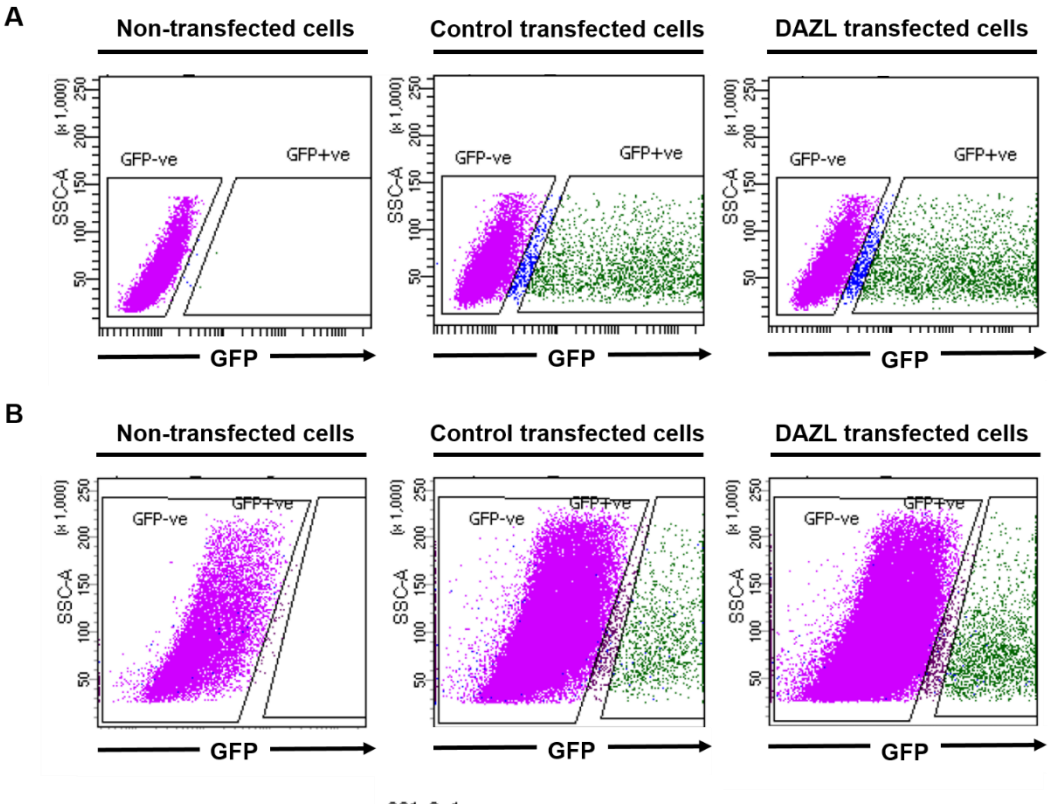


Figure 6.18. Cells could be enriched for presence of the DAZL or control plasmids by co-transfecting the (A) bovine and (B) human cells with a GFP plasmid. GFP-positive cells were collected using FACS. Control transfected and DAZL transfected cells exhibited similar transfection rates. Non-transfected cells were used to gate for GFP fluorescence.

Putative bovine OSCs demonstrated a wide range of transfection rates from 5.2% to 24.7%, with similar intra-experiment, but variable inter-experiment rates observed (Table 6.6). Transfection rates were similar regardless of whether the DAZL plasmid or the control plasmid had been utilised.

Table 6.6. Transfection rates of putative bovine OSCs according to passage number.

Passage Number	Transfection Rates (Mean ± S.E.M)
17	21.1% ± 0.6%
19	5.3% ± 0.3%

The enrichment of putative bovine OSCs was effective, with DAZL transfected cells demonstrating a significantly increased expression of *DAZL* mRNA compared with control transfected cells ($p < 0.01$ or < 0.001; Fig. 6.19; n = 5 or 6 per treatment group). *DAZL* expression was not affected by treatment with BMP4 and RA.

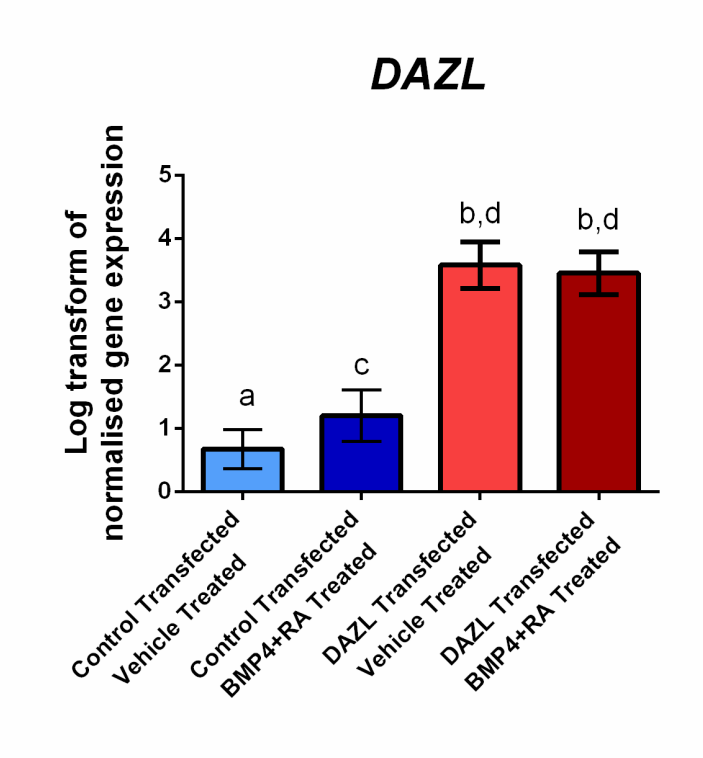


Figure 6.19. Enrichment for transfected putative bovine OSCs was effective, with DAZL transfected cells demonstrating significant upregulation of the mRNA. (a *vs*. b: $p < 0.001$, c *vs*. d: $p < 0.01$; mean ± S.E.M.; n = 5 or 6 replicates per treatment group performed during 2 separate experiments).

Analysis of the downstream genes demonstrated a pattern of increased expression with BMP4 and RA treatment of *ID1*, *MSX1* and *SYCP3* compared with vehicle-treated cells for both transfected groups, and in *MSX2* for the DAZL transfected cells only (Fig. 6.20). There was also a trend for *DAZL* transfected and BMP4 plus RA treated cells to have upregulated expression of *ID1*, *MSX2* and *SYCP3* compared with control transfected and BMP4 plus RA treated cells. However, none of these results were statistically significant and despite similar n numbers to the BMP4 and/or RA treatment experiments, the standard errors of the mean were larger, indicating less consistent results.

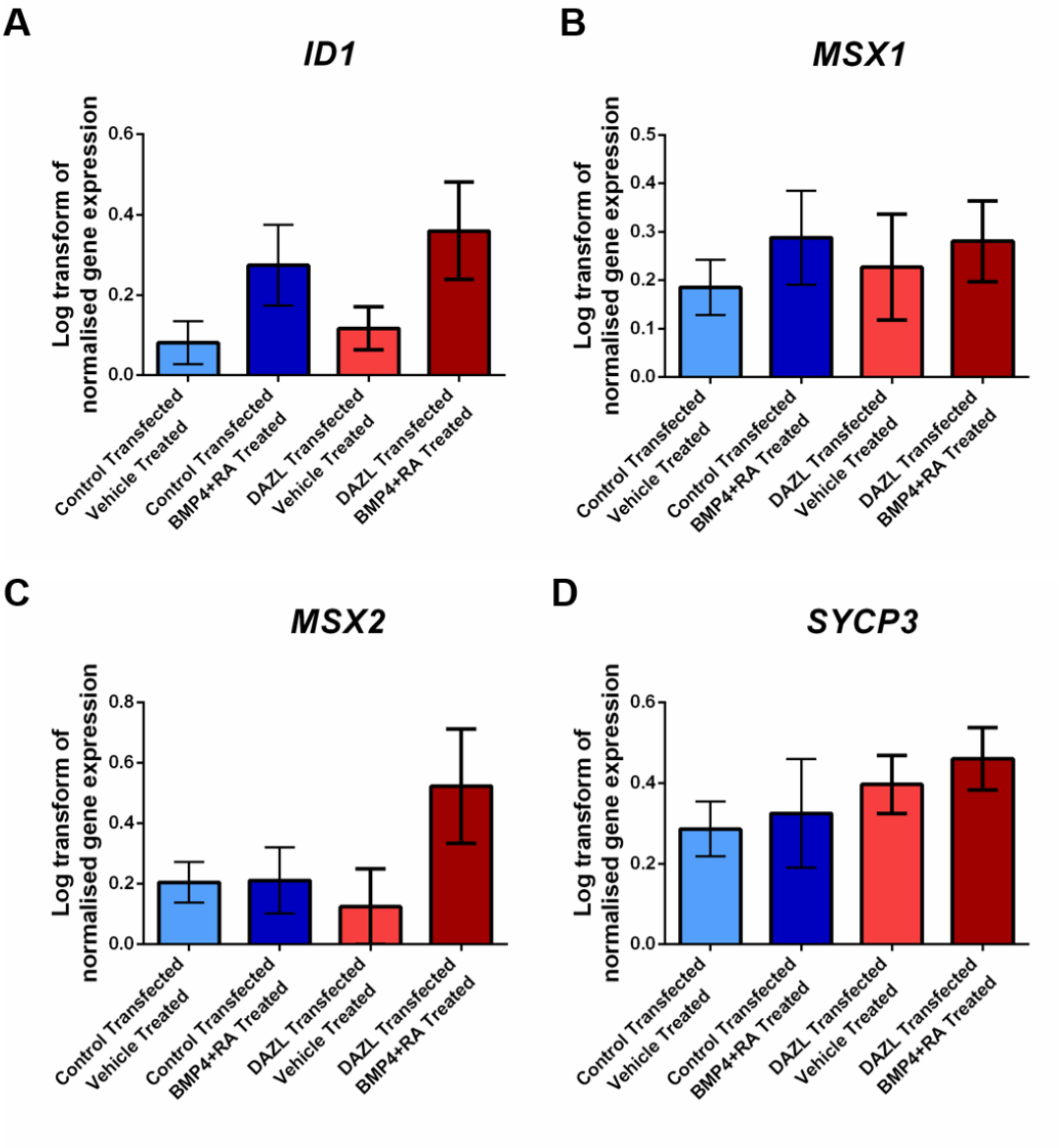


Figure 6.20. Expression of BMP4- and RA-response genes in enriched putative bovine OSCs that had been transfected with DAZL or control vectors and treated with vehicle or BMP4 plus RA. (A), **(B)**, and **(D)** *ID1*, *MSX1* and *SYCP3* expression exhibited a trend for increased expression with BMP4 plus RA treatment, which was only seen in DAZL transfected cells for *MSX2* **(C).** *ID1* and *SYCP3* appeared to be augmented by *DAZL* overexpression if the cells had been treated with BMP4 and RA, but this was not significant. (Mean ± S.E.M.; n = 5 or 6 replicates per treatment group performed during 2 separate experiments).

The experiment was repeated with putative human OSCs, which also demonstrated variable inter-experimental transfection rates, ranging from 1.5% to 16.4% (Table 6.7).

Table 6.7. Transfection rates of putative human OSCs according to passage number.

Passage Number	Transfection Rates (Mean ± S.E.M)
12	13.2% ± 1.3%
15	3.0% ± 0.2%

The enrichment for *DAZL*-expressing cells was again successful, with significant upregulation of the gene in DAZL plasmid-exposed cells ($p < 0.0001$; Fig. 6.21(a); n = 5 or 6 per treatment group). *ID1* was significantly upregulated with BMP4 and RA treatment in both transfected groups, however, DAZL transfected cells exhibited this increase in expression to a lesser degree than control transfected cells ($p < 0.05$ compared with $p < 0.001$; Fig. 6.21(b)). *RARβ* expression was also increased by BMP4 plus RA treatment, reaching significant levels when DAZL transfected, BMP4 and RA treated cells were compared with DAZL transfected, vehicle-treated cells ($p < 0.01$; Fig. 6.21(c)). Cells that had been both transfected with DAZL and treated with BMP4 plus RA also exhibited significantly upregulated levels of *RARβ* compared with control transfected, vehicle-treated cells ($p < 0.01$). *MSX1*, *MSX2* and *SYCP3* were undetectable.

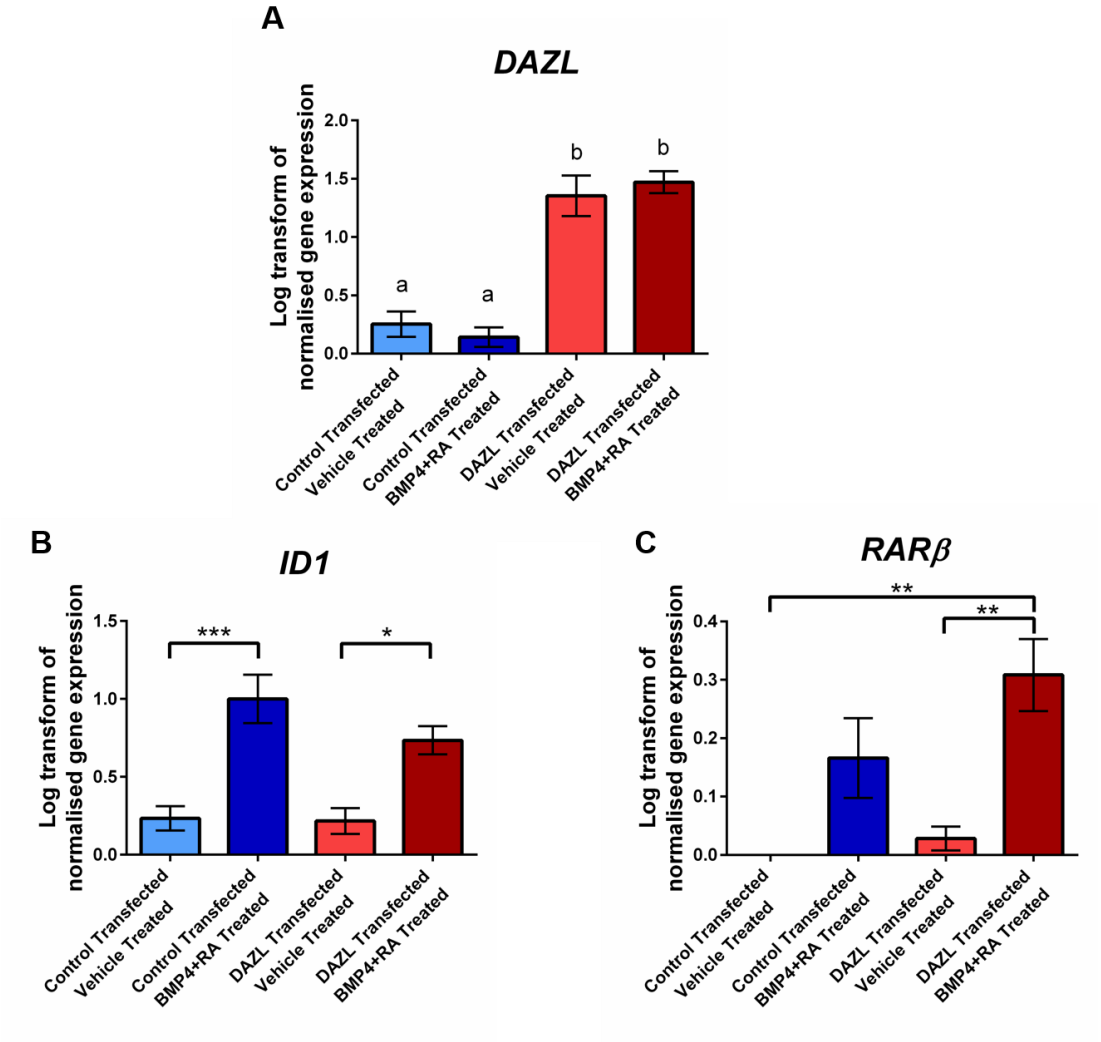


Figure 6.21. Enrichment for transfected putative human OSCs was also effective (A), with DAZL transfected cells demonstrating significant upregulation of *DAZL* mRNA. (a *vs.* b: $p < 0.0001$; mean ± S.E.M.; n = 5 or 6 replicates per treatment group performed during 2 separate experiments). (B) and **(C)** Analysis of BMP4- and RA-response genes in enriched human OSCs that had been transfected with DAZL or control vectors and treated with vehicle or BMP4 plus RA revealed significant upregulation of *ID1* with BMP4 and RA treatment, which was greater in control transfected cells compared with DAZL transfected cells. RARβ expression was significantly upregulated only in DAZL transfected and BMP4 plus RA treated cells. (*$p < 0.05$, **$p < 0.01$, *** $p < 0.001$; mean ± S.E.M.; n = 5 or 6 replicates per treatment group performed during 2 separate experiments).

6.3.2.3 DAZL protein expression

As the results of the enrichment experiments did not demonstrate significant downstream effects with increased *DAZL* transcription, Western blotting was performed to investigate whether the cells were capable of translating the *DAZL* mRNA to protein. A time course experiment for putative OSCs of both species was performed to investigate when protein expression was highest. DAZL expression was not confirmed in the bovine cells at any time point (Fig. 6.22; n = 1 replicate per treatment group); however, the human cells did demonstrate protein expression, which appeared to be highest 24 – 48 hours after transfection, before declining (Fig. 6.23; n = 1 replicate).

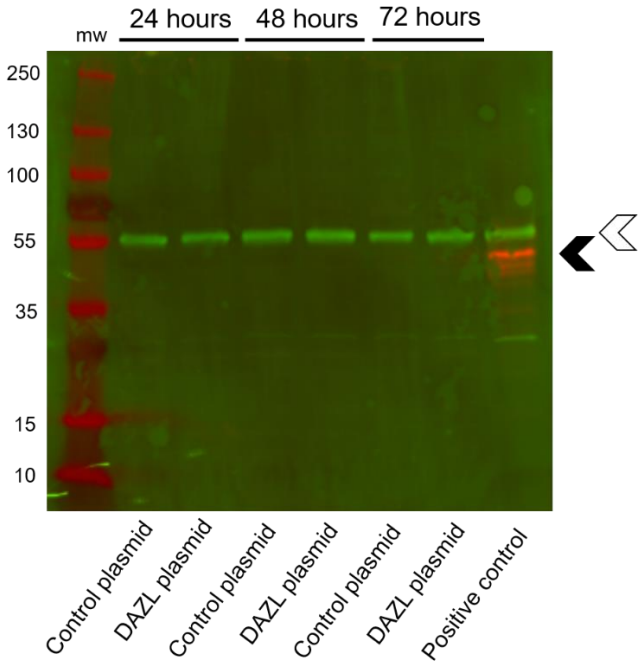


Figure 6.22. Western blot time course analysis for DAZL expression in putative bovine OSCs that had been exposed to DAZL plasmids or control plasmids. Probing of the cells failed to detect the protein, which was detected in the positive control (HEK-293 cells; black arrow). The expected molecular weight (mw) of DAZL is 38 kDa, however the Myc-DDK tag on the plasmid results in the band being located at a slightly higher mw. α-tubulin was used as a loading control (white arrow; expected mw 50 kDa).

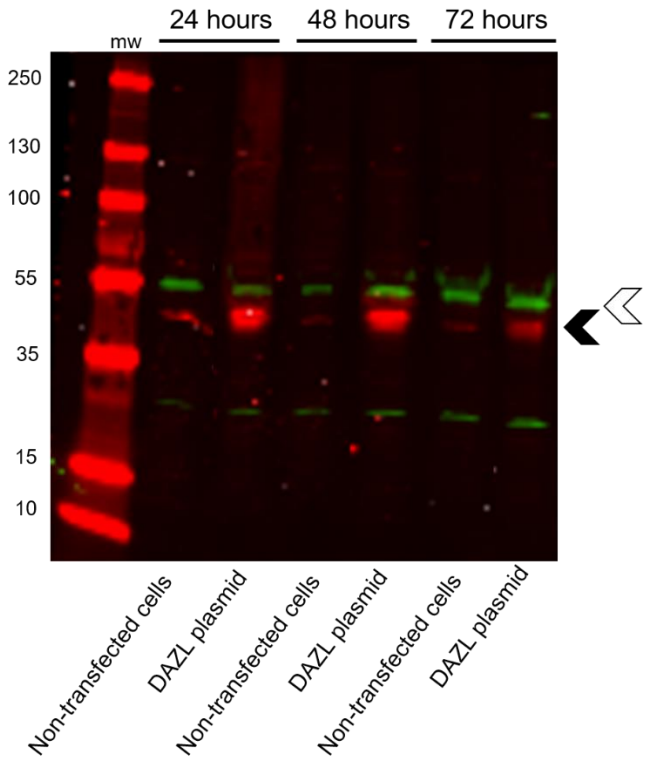


Figure 6.23. Western blot time course analysis for DAZL expression in putative human OSCs that had either been exposed to DAZL plasmids or not transfected. Non-transfected cells demonstrated low levels of endogenous DAZL, that was increased with DAZL transfection (black arrows; expected molecular weight (mw) 38 kDa, however a band of greater mw is seen due to the Myc-DDK tag on the plasmid). α-tubulin was used as a loading control (white arrow; expected mw 50 kDa).

6.4 Discussion

6.4.1 The effect of BMP4 and/or RA treatment on putative OSCs

The findings detailed in this Chapter demonstrate that bovine putative OSCs show promise as a germ cell model as their molecular response to two key regulators of the PGC development pathway, BMP4 and RA, was similar to that of germ cells, i.e. BMP4- and RA-response genes related to germ cell differentiation and meiosis initiation were upregulated. Firstly, putative bovine OSCs significantly upregulated their expression of *ID1* in response to BMP4. This is in keeping with both unpublished work from Prof. Anderson's group in human fetal ovaries and studies in mouse fetal gonads (Dudley *et al.*, 2007). A further enhancement of this response was observed with combined BMP4 plus RA treatment. Although the effect of RA on germ cell *ID1* expression has not been previously reported, the gene has been demonstrated to be RA-responsive in other cell types, including keratinocytes (Villano and White, 2006) and acute promyelocytic leukaemia cells (Nigten *et al.*, 2005).

MSX2 expression followed the same pattern as *ID1*. In contrast, *MSX1* was not upregulated by BMP4 alone: combined BMP4 and RA exposure was required for a significant increase in gene expression to be detected. Again, this is a similar response to that found in previous work using human fetal ovary, where BMP4 treatment selectively upregulated *MSX2*, but had no effect on *MSX1* (Childs *et al.*, 2010). It also supports mouse fetal ovary studies which demonstrated upregulation of *Msx2* in response to Bmp4 and increased *Msx1* expression in response to RA (Le Bouffant *et al.*, 2011). In contrast to the human fetal ovary study and the putative bovine OSC results, *Msx1* has been found to be BMP4-responsive in mouse fetal ovaries (Le Bouffant *et al.*, 2011): it is possible that inter-species differences may account for this incongruity. This further highlights the need for species-specific germ cell models, as findings from other species are not necessarily applicable to the species of interest.

Research on hESCs has demonstrated a link between BMP4 and *SYCP3*, with BMP4 treatment leading to an increased expression of *SYCP3* during the differentiation of hESCs into EBs (Kee *et al.*, 2006). RA has also been found to stimulate *SYCP3* expression in isolated mouse PGCs (Tedesco *et al.*, 2013). Similarly, our cultured

bovine cells demonstrated a significant upregulation of *SYCP3* following BMP4 treatment, with the addition of RA apparently augmenting this effect.

These mRNA effects were confirmed when the protein expression of ID1, MSX2 and SYCP3 was analysed immunocytochemically, with BMP4 plus RA-treated cells exhibiting increased expression, both qualitatively and quantitatively, of each of the three proteins when compared with vehicle-treated cells. SYCP3 expression was only seen in BMP4 plus RA-treated cells and exhibited an elongated staining, postulated by others to correspond to synaptonemal complexes at the zygotene, pachytene or diplotene stages of meiotic prophase I (Kee *et al.*, 2009, Medrano *et al.*, 2012).

If the putative OSCs were undergoing differentiation and meiotic entry, loss of pluripotency markers and increased expression of markers of meiotic germ cells may be expected. Indeed, analysis of hESC-derived EBs revealed downregulation of the pluripotency genes, *POU5F1* and *NANOG*, and successive upregulation of the germ cell markers, *DAZL* and *DDX4*, during the two week culture period (Aflatoonian *et al.*, 2009). This was accompanied by an increase in expression of the oocyte marker *GDF-9* and the marker of meiotic entry, *SYCP3*. The putative bovine OSCs demonstrated no significant changes in mRNA expression of *POU5F1*, *LIN28*, *IFITM3* or *C-KIT*, although a shorter culture period was used. However, this is in keeping with unpublished work from Prof. Anderson's group which has demonstrated that BMP4-treated human fetal ovaries do not exhibit changes in pluripotency (*POU5F1*) and germline (*DAZL* and *DDX4*) markers. In contrast, our experiments demonstrated that *PRDM1* expression was upregulated by combined BMP and RA treatment. This finding is of note, as in mice Bmp4 has been shown to stimulate epiblast cells to express *Prdm1* and cause germ cell specification (Ohinata *et al.*, 2009). The upregulation of the gene in the bovine cells may therefore reflect differentiation of the cells into PGCs.

The human data were not as robust as the bovine experiments, with the *ID1* response to BMP4 and/or RA treatment being the only finding conserved between the two species. *MSX1* again appeared to be RA-responsive and *SYCP3* demonstrated a trend for increased expression, but these results were not significant. Higher n numbers may help to elucidate the gene expression effects. The cells did, however, upregulate the

expression of the RA receptor, *RARβ*, when treated with RA alone or combined BMP4 and RA treatment, demonstrating their RA-responsiveness. The gene was not responsive to BMP4 treatment alone, indicating the increased levels in response to combined treatment were only due to the presence of RA. Unlike the bovine cells, analysis of the proteins of interest did not reveal differences in ID1 and MSX2 expression between vehicle-treated and BMP4 plus RA-treated cells. It was noteworthy that MSX2 protein was detectable, yet *MSX2* mRNA was not. This may have been a technical issue with the *MSX2* primers, although they had been previously validated for use on human fetal ovary (Childs *et al.*, 2010). No SYCP3 expression was seen in any cells. It would appear, therefore, that mRNA changes were not translated to protein expression changes in the human cells. This may, once again, be due to inter-species differences, or it may be that the human cells required a longer period of treatment than the bovine cells for translation to occur and an effect to be observed, or that they require additional factors not present in these experiments.

PGCs are not the only cell type that demonstrate BMP4 and RA-responsiveness, with multiple roles established in many different tissues (Hogan, 1996, Rhinn and Dolle, 2012, Katagiri and Watabe, 2016); however, germ cells are the only cell type with the ability to undergo meiosis. BMP4 and/or RA treatment experiments were therefore performed on fetal bovine somatic cells to assess whether the putative OSCs demonstrated a distinct molecular response compared to ovarian somatic cells. The somatic cells appeared to be BMP4-responsive, with increased expression of *ID1*, *MSX1* and *MSX2*. Although none of the changes in expression reached significance, this may be due to an insufficient sample size. Importantly, the cells did not demonstrate an increase in gene expression with treatment of classic markers of meiotic entry, with no significant changes in expression of *SYCP3* and *STRA8* detected. Again, no changes in ID1 and MSX2 protein expression with BMP4 plus RA treatment were observed and no cells expressed SYCP3.

Overall, these results demonstrate that the bovine putative OSCs robustly mimic the molecular response of PGCs to two key regulators of germ cell development, including upregulation of the expression of the meiotic entry gene, SYCP3, at both the mRNA and protein level. This is not observed in fetal bovine ovarian somatic cells. Thus, the

putative bovine OSCs demonstrate promise as a model for germ cell development. As a large animal, this could prove valuable in providing a more translatable model to explore the intricacies of human female germ cell development, as well as providing a bovine-specific germ cell model. Currently, derivation of bovine ESCs is proving problematic: derived ESC-like cells fail to maintain an undifferentiated state and have not been shown to proliferate long term (Maruotti *et al.*, 2012, Hall *et al.*, 2013). Therefore, putative bovine OSCs may provide an alternative option for examining bovine germ cell development. It should be emphasised, however, that while SYCP3 is a hallmark of entry into meiosis, relevant chromosomal changes were not explored thus it remains unclear whether these cells can in fact enter meiosis. Nevertheless, these findings corroborate those of Park *et al*., who are the only group to publish data on OSCs and BMP4 treatment (Park *et al.*, 2013). Their research utilising mouse OSCs also demonstrated upregulation of *Msx1*, *Msx2* and another classis marker of meiotic entry, *Stra8*. However, in contrast to the murine experiments (Park *et al.*, 2013), no OLCs were observed during the bovine and human investigations.

6.4.2 The effect of overexpression of *DAZL* on putative OSCs

Following the BMP4 and/or RA treatment experiments, we postulated that an increase in *DAZL* expression may further augment the expression of meiotic markers in putative bovine OSCs and elucidate the *SYCP3* treatment response in the human cells. DAZL was selected as (a) it is essential for germ cells to enter meiosis, with *Dazl* deficient mice exhibiting significantly reduced levels of several genes related to meiosis (Lin *et al.*, 2008), (b) overexpression of the gene has been shown to induce differentiation of hESCs and iPSCs into PGCLCs (Medrano *et al.*, 2012) and (c) Sycp3 has been demonstrated to be a Dazl target in mice (Saunders *et al.*, 2003, Reynolds *et al.*, 2007). In order to generate *DAZL* overexpression in putative bovine and human OSCs, a liposomal-mediated transfection technique was utilised. This method of transfection has previously been performed in rodent OSCs, to both silence (Zhang *et al.*, 2011) and cause overexpression (Zhou *et al.*, 2014) of specific genes, generating transgenic offspring. To our knowledge, it has never been utilised in the setting of investigating OSCs regarding germ cell model potential.

DAZL transfection of the bovine and human cells was rapidly effective with a significant increase in *DAZL* mRNA expression in DAZL transfected cells compared to the very low levels of endogenous *DAZL* detected in control transfected cells. This low level of endogenous DAZL expression was also demonstrated at the protein level in non-transfected human cells, with transfection successfully resulting in increased protein levels. Preliminary experiments on bovine and human cells demonstrated that DAZL transfection alone did not affect the expression of the downstream genes, *ID1*, *MSX1*, *MSX2* (detected in bovine experiments only) and *SYCP3*. When human cells were both transfected and then treated with BMP4 plus RA, the expected *ID1* and *RARβ* response to BMP4 plus RA was observed in both control and DAZL transfected cells, although this did not reach significance, possibly due to low n numbers. No significant augmentation of expression of any of the analysed genes was seen in the DAZL transfected, BMP4 plus RA treated human cells compared with control transfected cells, although *SYCP3* expression in DAZL transfected, BMP4 plus RA treated cells was higher than the other treatment groups.

Co-transfection of the cells with DAZL/control plasmid and GFP plasmid allowed transfection rates to be estimated by flow cytometry, with low and variable rates detected. Given the standardised transfection methodology and the use of the same bovine and human cell lines in the experiments, it is possible that the cause of the variations in transfection rates was due to heterogeneity of the cells at differing passages. Indeed, there was an observed association between lower transfection rates and later passages in both species. This phenomenon has been observed in other cell lines: HEK-293 cells exhibit differing transfection rates between lower and higher passages depending on the serum content of the culture medium, with serum-free medium demonstrating lower transfection rates at greater passages (de Los Milagros Bassani Molinas *et al.*, 2014). In our protocol, cells were initially transfected in serum-containing medium, before being changed to serum-free medium for BMP4 plus RA treatment, therefore the changing serum content may have had an effect on transfection efficiency. Human cells appeared to be less readily transfected than bovine cells, as human cells at passage 15 exhibited lower transfection rates than bovine cells at P17. This is in keeping with the lentiviral transduction rates detailed in Chapter 3 and may be due to interspecies differences in susceptibility to plasmid uptake.

Enrichment for transfected cells by flow cytometry was successful, allowing analysis of a purer population of DAZL (or control) transfected cells, thus removing the "dilution" effect of large numbers of non-transfected cells that were present in the initial experiments. The BMP4 plus RA treatment response appeared conserved in enriched DAZL and control transfected cells of both species, although only *ID1* and *RARβ* expression in the human cells reached significant levels. This observed blunting of the treatment effect seen in non-transfected cells may be due to less consistent results in the transfection experiments (indicated by larger S.E.Ms). One possible reason for this is that poor transfection rates resulted in low numbers of enriched cells and thus low total RNA concentrations for qRT-PCR analysis, making robust analysis of the genes, especially lowly expressed genes, difficult. This is supported by the finding that *MSX1* was lowly detected in the non-transfected and non-enriched human experiments, but was never detectable in the enriched experiments. Furthermore, transfection may have been detrimental to the cells' ability to respond to BMP4 plus RA treatment: GFP transduction of the cells has already been demonstrated to be detrimental to cell health in Chapter 3 and perhaps translation of GFP was too energy-demanding for transcription of other genes to occur.

Yet, cells that were both DAZL transfected and BMP4 plus RA treated did demonstrate increased expression of *ID1*, *MSX2* and *SYCP3* compared with control transfected and BMP4 plus RA treated cells in the bovine experiments, although significant changes were not detected. In contrast, DAZL transfected human cells actually demonstrated lower levels of *ID1* upregulation than control transfected cells after BMP4 plus RA treatment. Similar to the GFP hypothesis, perhaps the high levels of *DAZL* transcription generated by the overexpression plasmid was injurious to the ability of the human cells to respond to treatment. The only gene where DAZL transfection appeared to have a significant effect on expression was *RARβ* in the human experiments. This is in keeping with mouse studies which demonstrated that Dazl enables PGCs to respond to RA for meiosis to be initiated (Lin *et al.*, 2008). Yet, both DAZL transfection and BMP4 plus RA treatment had to be performed for the cells to significantly upregulate their expression: although there was a trend for increased expression in DAZL transfected cells compared with control transfected cells when the two BMP4 plus RA treatment groups were compared, this was not significant. Our

sample sizes were limited by cell availability, however; a greater sample size would improve the power of the experiment.

Lastly, it is of note that, in contrast to human cells, DAZL protein expression was not detectable in bovine cells. As the same plasmid and Western blotting antibody were used in both species and non-enriched cells were utilised, it is possible that the levels of DAZL protein were simply too low in the bovine cells to be detectable by Western blotting. The hypothetical stimulatory effect of downstream genes in the presence of elevated levels of DAZL may therefore not have been observed due to inadequate DAZL expression. Yet, this does not explain why human cells, which did exhibit increased DAZL expression after DAZL transfection, did not demonstrate significant downstream effects due to DAZL transfection alone.

Overall, the findings did not prove the hypothesis that *DAZL* overexpression results in increased expression of downstream meiosis-related genes, although limitations with regards inefficient transfection rates and low numbers of transfected cells may have contributed to the inconclusive results.

6.4.3 Summary

In vitro approaches do not address the fundamental question of the physiological relevance of OSCs, but do provide an opportunity to explore germ cell development in ways that are not possible *in vivo* in large animals and humans. Experiments aimed at exploring the potential of putative bovine and human OSCs as a model of germ cell development demonstrated that the bovine cells showed changes in downstream genes, including meiosis-related genes, in response to key regulators of the development pathway, in keeping with the findings reported in fetal ovary and PGC research in the literature. Putative human OSCs showed similar trends in gene response patterns; however, the results were not as conclusive. Overexpression of a critical gene related to meiotic entry, DAZL, did not have significant stimulatory effects on downstream genes. The effects of either RA exposure or DAZL overexpression on OSCs have not been investigated previously and are therefore novel approaches in the investigation of the potential of such cells as germ cell models. Clearly, mRNA expression of genes critical to meiosis initiation does not equate to the cells truly entering meiosis and

therefore future work should focus on examining the chromosomal changes that occur within these cells in response to BMP4 and/or RA treatment

Chapter 7

A Novel OSC Delivery System

7.1 Introduction

7.1.1 The delivery of OSCs to the ovary for clinical applications

As discussed in section 1.1.3, an exciting possible role of OSCs in clinical Medicine could be in the area of fertility preservation. One proposed technique by which OSCs could be utilised is the isolation of the cells from an ovarian biopsy prior to POI-inducing treatment, proliferation of the cells *in vitro* and then injection of the cells back into the woman's ovaries following disease remission (Dunlop *et al.*, 2013). Once *in situ*, the OSCs could potentially produce new oocytes *in vivo* and restore a woman's reproductive and ovarian endocrine function, at least temporarily. However, as detailed in Chapter 5, injections of cells into ovarian tissue can be technically difficult, with the risk of cell extrusion into the surrounding environment. This may be especially true if injections are performed laparoscopically, when adequately immobilising the ovary for efficient injection could prove problematic. An acellular scaffold could provide an alternative method of cell delivery: insertion of the scaffold, seeded with OSCs, into the ovarian medulla via an incision could be performed laparoscopically, in a similar manner to the orthotopic reimplantation of cryopreserved ovarian cortex during fertility preservation techniques (Sanchez-Serrano *et al.*, 2010, Donnez *et al.*, 2013). Scaffolds have been proposed to allow greater retention of transplanted cells within the desired location than injection techniques (Damous *et al.*, 2015).

The use of artificial, or engineered, ovaries utilising scaffolds to both mature oocytes *in vitro* and transplant isolated follicles and ovarian somatic cells for *in vivo* oocyte maturation is currently an emergent area of research. This approach would be especially valuable in women with haematological malignancies as reimplanting cryopreserved ovarian cortex in these patients carries the risk of reintroducing malignant cells (Dolmans *et al.*, 2010, Rosendahl *et al.*, 2010). Materials used to create 3D artificial ovaries include agarose honeycomb-shaped moulds (Krotz *et al.*, 2010), alginate and alginate-matrigel matrices (Vanacker *et al.*, 2012, Laronda *et al.*, 2014, Vanacker *et al.*, 2014), fibrin clots (Luyckx *et al.*, 2013, Luyckx *et al.*, 2014, Kniazeva *et al.*, 2015, Soares *et al.*, 2015, Chiti *et al.*, 2016, Paulini *et al.*, 2016), decellularised ovaries (Laronda *et al.*, 2015) and a gelatin scaffold created by a 3D printer (Laronda *et al.*, 2016). The majority of studies have been performed in mice by Amorim and colleagues in Belgium, utilising an *in vivo* technique that comprises seeding of a fibrin or alginate-containing scaffold with isolated immature murine follicles and additional

supportive ovarian cells before subsequent autografting of these structures into recipient mice (Vanacker *et al.*, 2012, Luyckx *et al.*, 2014, Vanacker *et al.*, 2014, Chiti *et al.*, 2016). This has resulted in healthy follicle growth after 1 week of grafting, as assessed by TUNEL staining for apoptosis and staining for the proliferative marker, Ki67. The group extended this research into humans, with fibrin-encapsulated isolated human preantral follicles demonstrating growth and viability after being xenografted into nude mice for 1 week (Paulini *et al.*, 2016). However, although growth to the antral stage in mice (Vanacker *et al.*, 2014) and to secondary follicles in humans (Paulini *et al.*, 2016) has been reported, the complete maturation and fertilisation competence of the oocyte was not demonstrated in these studies.

Using an agarose mould as a scaffold, *in vitro* maturation of human oocytes from early antral follicles has been reported, following seeding of the moulds with theca cells and COCs (Krotz *et al.*, 2010). After 3 days, the COCs were surrounded by theca cells and one (of three) oocytes had undergone polar body extrusion. However, the sample size was clearly very small and, again, developmental competence of the oocyte was not tested. Furthermore, if such a technique was to be used clinically, then efficient maturation from the primordial stage would be necessary to maximize the number of competent oocytes obtained. Another group at the forefront of this research, Woodruff and colleagues in the USA, have reported both resumption of endocrine function and livebirths in mice using a variety of scaffolds and isolated follicles, including those at the primordial stage (Kniazeva *et al.*, 2015, Laronda *et al.*, 2015, Laronda *et al.*, 2016). Decellularised bovine ovary scaffolds, seeded with ovarian cells from mice and grafted under the kidney capsule, supported the development of large antral follicles and the initiation of puberty in ovariectomised mice (Laronda *et al.*, 2015) and autografted fibrin-embedded immature murine follicles also resulted in the resumption of ovarian function (Kniazeva *et al.*, 2015). Furthermore, if the fibrin contained vascular endothelial growth factor (VEGF), the recipient mice produced live pups (Kniazeva *et al.*, 2015), likely due to improved vascularisation, and thus survival, of the graft. Most recently, a 3D printed gelatin scaffold devised by the group was reported to similarly support the production of live pups when seeded with isolated follicles and supportive ovarian cells (Laronda *et al.*, 2016). Similar principles could be applied to deliver OSCs directly into the ovary, or to transplant heterotopically both OSCs and supportive ovarian somatic cells for *in vivo neo*-oogenesis in women who have undergone bilateral oophorectomies. The biomaterial utilised in the experiments

detailed in this Chapter was gelatin, in the form of a clinically available product called Gelfoam®.

7.1.2 Gelfoam® as a cell delivery system

Gelfoam® is a gelatin sponge, derived from porcine skin, with a well-established clinical use in haemostasis (Jenkins *et al.*, 1946, Smith, 1947). It is a biocompatible, porous, flexible matrix, which can take up fifty times its weight in water and is absorbed by soft tissues within four to six weeks (Jenkins *et al.*, 1946, Smith, 1947). When placed on bleeding surfaces, it liquefies in one to four days (Jenkins *et al.*, 1946). These are valuable properties for a cell scaffold as (a) the sponge can absorb a large volume of cell suspension and (b) the cells can be gradually delivered to the desired area during liquefaction or absorption of the sponge. Several studies have now used Gelfoam® for this purpose: it has shown promise as a scaffold for bone marrow mesenchymal stem cells (MSCs) to enable cartilage regeneration (Ponticiello *et al.*, 2000) and enhance bone allograft healing (Lee *et al.*, 2011), and has been used to regenerate the right ventricular outflow tract in rats using fetal cardiomyocytes (Sakai *et al.*, 2001). Most relevantly, it has been used as a scaffold to deliver adipose tissue-derived stem cells (ASCs) to ovaries in rats (Damous *et al.*, 2015). Intra-ovarian injections of ASCs have previously been reported to improve ovarian function in mice after chemotherapy treatment, as assessed by follicle numbers and ovulated oocytes (Sun *et al.*, 2013). When ASC-seeded Gelfoam® was applied to frozen-thawed rat ovaries which were reimplanted into the retroperitoneum of oophorectomised rats in an attempt to restore ovarian function, the animals recommenced estrous cycles sooner than non-ASC controls, although no effect on follicle numbers or health was detected (Damous *et al.*, 2015) thus the mechanism of this apparent beneficial effect remains unknown at present.

7.1.3 Aims of this chapter

The aim of the experiments described in this Chapter was to perform a preliminary investigation of the potential of Gelfoam® as a cell delivery scaffold for OSCs. Although it has been utilised for the delivery of other types of stem cell, this would be a novel cell delivery approach for OSCs. The objectives of the experiments were to (a) assess whether Gelfoam® could be seeded successfully with putative bovine OSCs

and (b) explore whether cell-seeded Gelfoam® could effectively deliver the cells into bovine ovarian cortex.

7.2 Materials & Methods

7.2.1 Fluorescent labelling of putative bovine OSCs

Putative bovine OSCs (Cell Line 4, varying passages) were labelled with rhodamine-conjugated dextrans as per section 2.5.2. Cells were analysed using an inverted microscope (Axiovert 200) prior to use in experiments to ensure fluorescence was detectable (detailed in section 2.5.2).

7.2.2 Cell seeding of Gelfoam®

Gelfoam® sheets (Pharmacia and Upjohn Company) were cut into 1cm x 1cm x 0.3cm squares under sterile conditions and hydrated in OSC culture medium (see section 2.4) in 24 well culture plates for 1 hour prior to cell seeding. Rhodamine-labelled putative bovine OSCs were trypsinised, centrifuged at 800 x g for 5 mins and resuspended in 100µl of fresh OSC culture medium. The culture medium was then adjusted so the top of the Gelfoam® was just above the surface of the medium and the bovine cells were pipetted onto the top of the Gelfoam®. Cells from one confluent well of a 12 well plate was seeded onto each square (i.e. approximately 60000 cells). The seeded Gelfoam® squares were then incubated at 37ºC / 5% CO_2. Two sponges were cultured for 7 days, with fresh OSC medium added on alternate days so that the surface level of the medium was maintained just below the top surface of the Gelfoam®. These pieces were then fixed in 4% NBF for 24 hours. Three sponges were utilised in cell delivery experiments after only 24 hours of culture.

7.2.3 Cell delivery experiment

Cryopreserved bovine ovarian cortical pieces were removed from liquid nitrogen storage and thawed as per section 2.2.2. The pieces were cultured in McCoy's 5A medium with supplements (see section 5.2.1.1) in 24 well plates at 37ºC / 5% CO_2 for 24 hours prior to use. The following day, the pieces were removed from culture and placed in a glass Petri dish in a laminar flow hood. One piece was placed on a sterilised stainless steel acupuncture needle with fine forceps. A seeded Gelfoam® square was

then placed on the needle, before a second ovarian cortical piece was threaded onto the needle, thus "sandwiching" the Gelfoam® square (Fig. 7.1). The cortical pieces were placed on the needle so that the OSE was on the outside of the "sandwich", thus ensuring the Gelfoam® was in contact with the cut edges of the innermost cortex. The needle was trimmed with sterile scissors and the "sandwich" was placed with the needle orientated vertically in one well of a 24 well plate containing fresh OSC culture medium, then incubated at 37ºC / 5% CO_2 for 7 days before being fixed in 4% NBF for 24 hours.

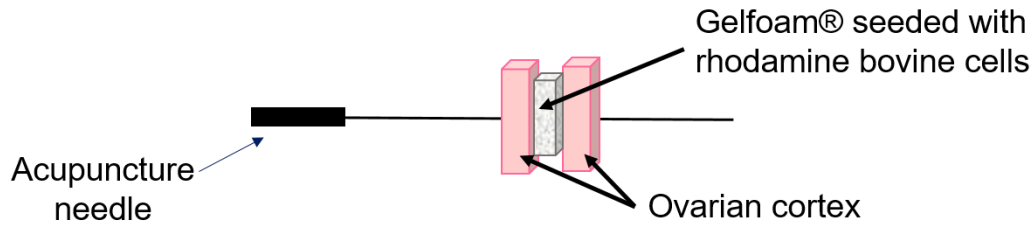


Figure 7.1. Schematic of the ovarian cortex/Gelfoam® "sandwich" utilised in the cell delivery experiments.

7.2.4 Haematoxylin and eosin staining

Fixed cell-seeded Gelfoam® and cortex/Gelfoam® "sandwiches" were embedded at SuRF and serial sectioned as per section 2.6.1. Haematoxylin and eosin staining was then performed as per sections 2.6.2 and 2.6.3. Slides were analysed and images were acquired as detailed in section 2.8.1.

7.2.5 Immunofluorescence

Every 3rd slide of cell-seeded Gelfoam® and cortex/Gelfoam® "sandwiches" was analysed by IF for the presence of rhodamine-labelled cells. Slides were dewaxed and rehydrated as per section 2.6.2, counterstained with DAPI (1:1000, diluted in PBS) and mounted with PermaFluor™ as per section 5.2.2.4. Slides were analysed and images were acquired as per section 2.8.2. For negative controls, non-seeded Gelfoam® or a "sandwich" containing Gelfoam® seeded with non-rhodamine labelled cells was utilised.

7.3 Results

7.3.1 Cell seeding of Gelfoam®

Putative bovine OSCs were labelled with rhodamine-conjugated dextrans (Fig. 7.2) and seeded into Gelfoam® squares (Fig. 7.3(a)). Cells could not be detected by haematoxylin and eosin staining alone (Fig. 7.3(b)), but were detectable by IF, with punctate cytoplasmic rhodamine staining visible (Fig. 7.4).

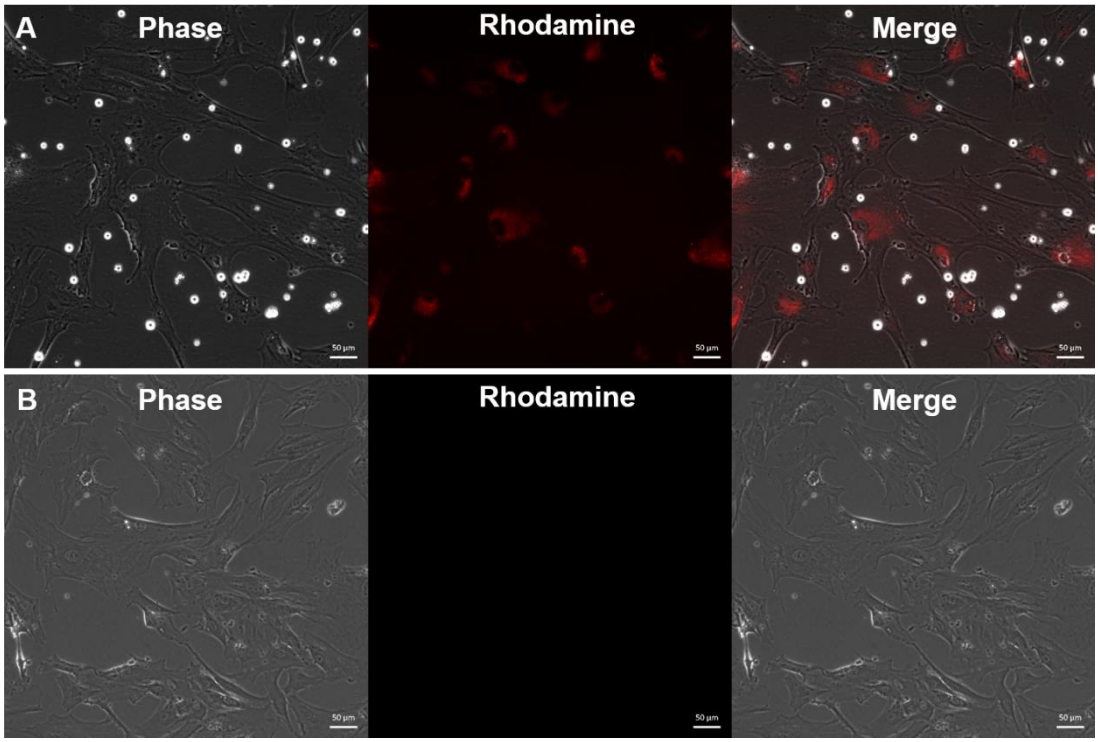


Figure 7.2. Putative bovine cells were fluorescently labelled with rhodamine-conjugated dextrans prior to seeding on Gelfoam®. (A) Rhodamine-labelled cells, adherent to the plate, were clearly visible after dextrans exposure. The white circular cells visible were non-adherent and non-viable. **(B)** Non-labelled cells were used as a negative control. Scale bar = 50µm.

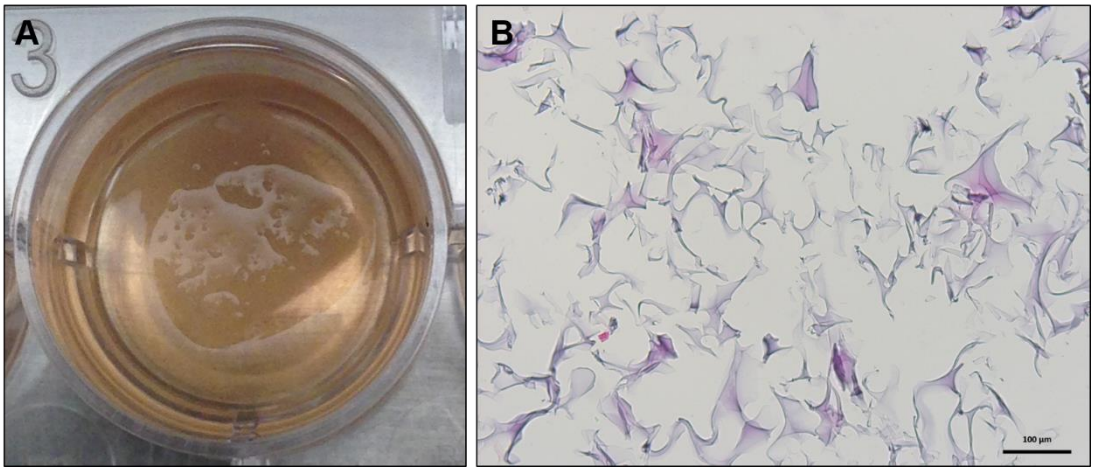


Figure 7.3. Putative OSC-seeded Gelfoam®. (A) Appearance of cell-seeded Gelfoam® within OSC culture medium after 7 days of culture in a 24 well plate. Prior to being placed in the medium, the Gelfoam® was sponge-like with a porous structure; once equilibrated in medium, it became more gelatinous in nature. **(B)** Haematoxylin and eosin staining demonstrates the structure of Gelfoam® but putative OSCs were not detectable. Scale bar = 100µm.

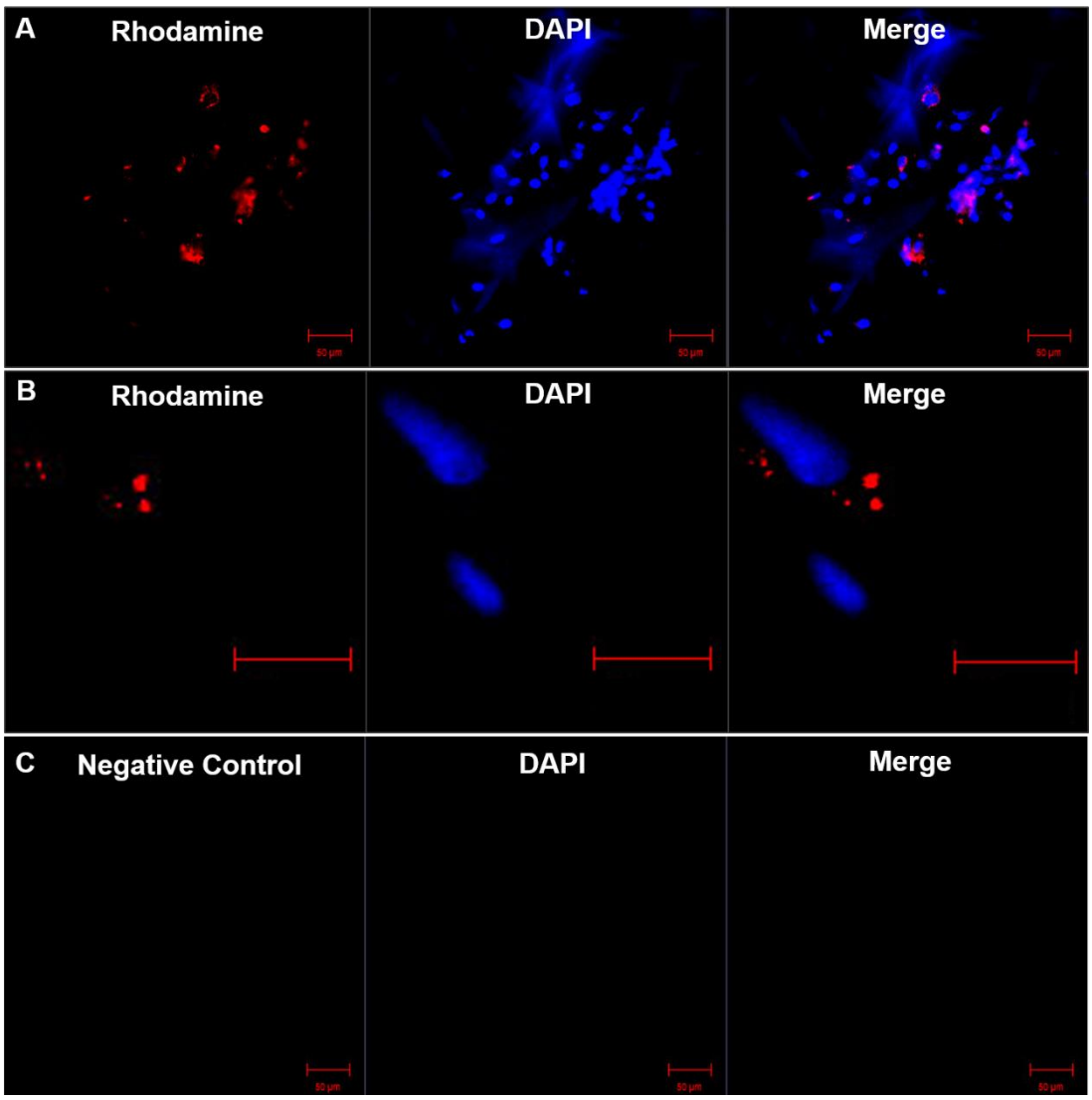


Figure 7.4. IF images of putative OSC-seeded Gelfoam®. (A) Cells were visible within the matrix, either by the presence of nuclei stained with DAPI, or rhodamine fluorescence. Not all cells were rhodamine-positive. Scale bar = 50µm. **(B)** An enlarged image of a rhodamine-positive cell demonstrating typical punctate cytoplasmic expression. Scale bar = 20µm. **(C)** Non-seeded Gelfoam® was used as a negative control. Scale bar = 50µm.

7.3.2 Gelfoam® as a cell delivery system

Ovarian cortex/Gelfoam® "sandwiches" were constructed (Fig. 7.5), which allowed close contact between the raw surface of the cortex and the seeded Gelfoam®.

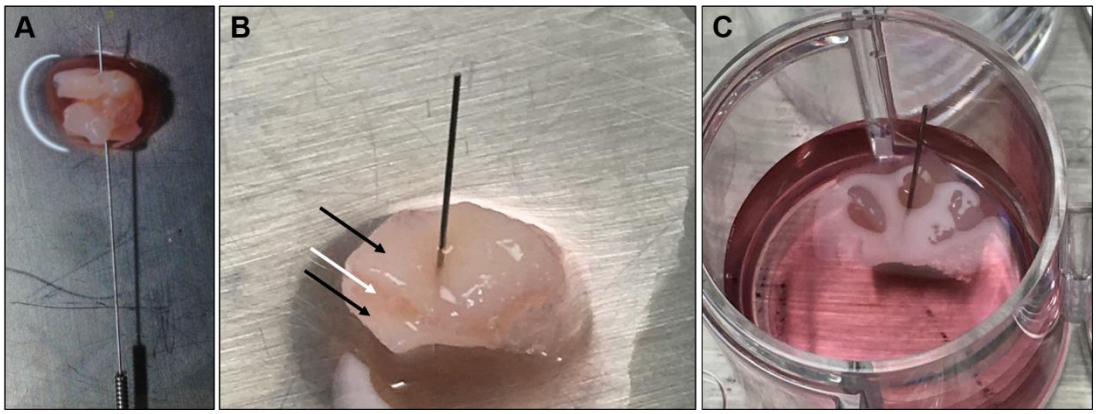


Figure 7.5. Images of the bovine ovarian cortex/Gelfoam® "sandwich". (A) The pieces of cortex and cell-seeded Gelfoam® were threaded onto an acupuncture needle. **(B)** The acupuncture needle was then trimmed and the "sandwich" orientated so that the needle was vertical. Black arrows = ovarian cortex, white arrow = Gelfoam®. **(C)** The orientation of the "sandwich" within a well of a 24 well plate.

Haematoxylin and eosin staining allowed the histological structure of the "sandwich" to be visualised (Fig. 7.6). Unhealthy, immature follicles were visible in the cortex, as assessed by morphology, with oocytes demonstrating poor association with surrounding pyknotic granulosa cells. IF revealed that rhodamine-labelled cells were detectable in the Gelfoam®, including at the cortex/Gelfoam® interface, but none were detected in the cortex (Fig. 7.7).

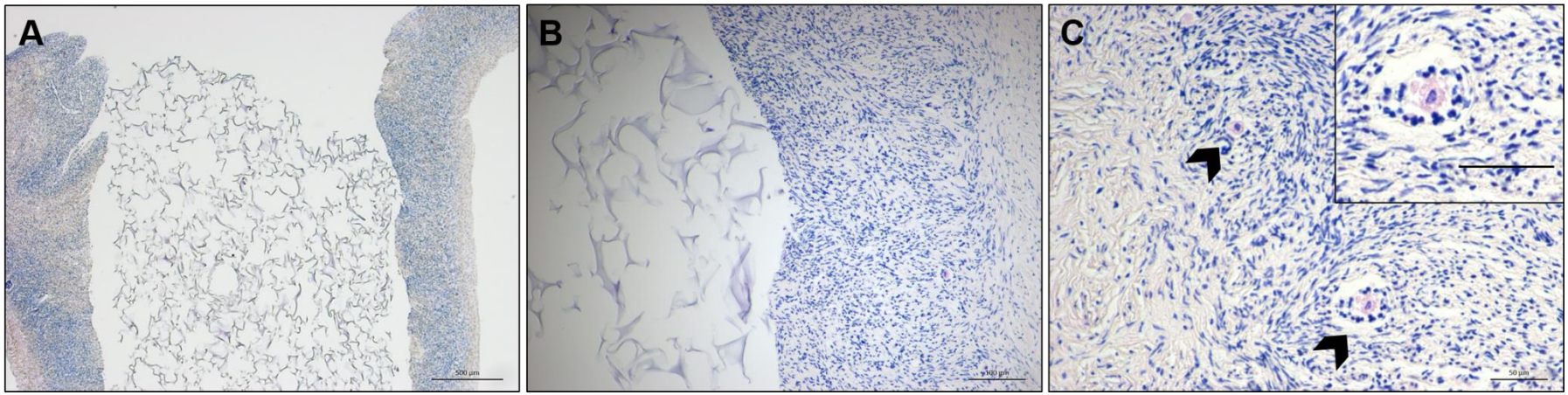


Figure 7.6. Haematoxylin and eosin staining demonstrating the structure of ovarian cortex/Gelfoam® sandwiches. (A) The Gelfoam® was in close contact with the innermost aspects of the ovarian cortex. Scale bar = 500µm. **(B)** An enlarged image of the interface shows the Gelfoam® immediately juxtaposed to the ovarian cortex. Scale bar = 100µm. **(C)** Follicles (black arrows) within the cortex were unhealthy as assessed by morphology. An enlarged image of the lower follicle is shown in the black box. Scale bars = 50µm.

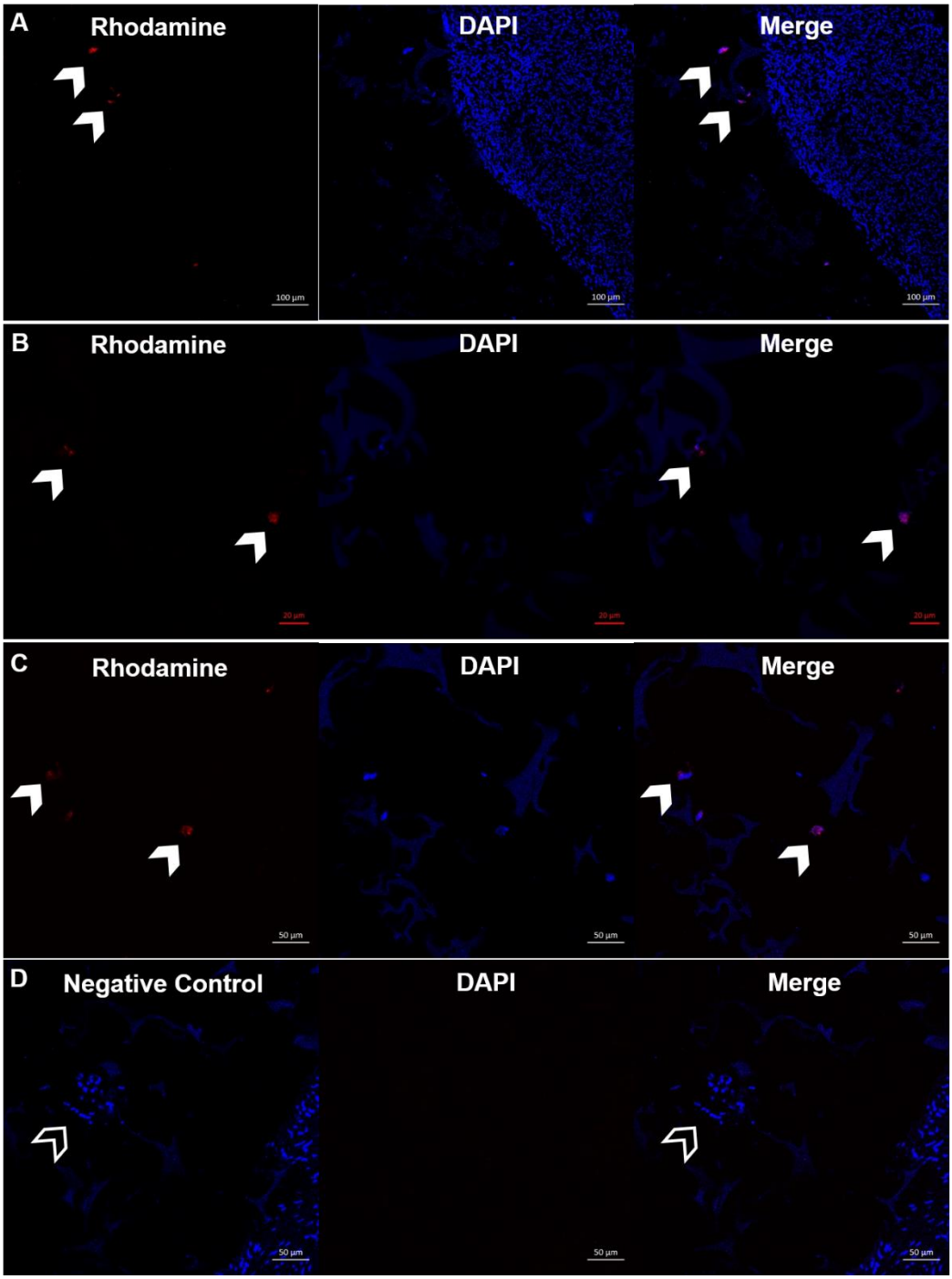


Figure 7.7. IF images of ovarian cortex/Gelfoam® "sandwiches". (A – C) Rhodamine-positive cells (white arrows) were observed within the Gelfoam®, including at the cortical/Gelfoam® interface; however, no rhodamine-positive cells were seen within the cortex. **(D)** A "sandwich" with non-rhodamine cells was used as a negative control. Cells are visible in the Gelfoam®, as detected by their nuclei (black arrows). Scale bars = 100µm **(A)**, 20µm **(B)** and 50µm **(C, D)**.

7.4 Discussion

7.4.1 The use of Gelfoam® as a cell scaffold

The results of these preliminary investigations into the use of Gelfoam® as an OSC delivery strategy demonstrate that putative bovine OSCs can be retained within the sponge for at least 7 days, being easily detectable by DAPI staining and rhodamine fluorescence. A study utilising ASCs demonstrated that the cells remained viable throughout a similar *in vitro* culture period of 5 days (Damous *et al.*, 2015). For the cell delivery experiments, the Gelfoam® was only seeded overnight, in an attempt to ensure adequate cell attachment to the matrix but avoid excessive cell loss through the porous structure that was hypothesised may happen during a longer seeding time period. This is in keeping with the short time frames of other studies: Damous *et al*. only seeded the Gelfoam® with ASCs for 10 mins prior to grafting (Damous *et al.*, 2015), whilst Lee *et al*. seeded and grafted the Gelfoam® on the same day (Lee *et al.*, 2011). In contrast, cardiomyocytes were seeded onto the scaffold for 1 to 3 weeks to allow intra-matrix *in vitro* expansion prior to engraftment (Sakai *et al.*, 2001). Given the paucity of published research on the use of Gelfoam® for cell delivery, it is perhaps unsurprising that a standardised protocol for its use is not available.

Our experiments focused on *in vitro* assessment of cell delivery as neither allotransplantation or xenografting of the putative OSC-seeded Gelfoam® was feasible due to lack of access to the required animals. Due to its structure, it would be technically challenging to make the Gelfoam® pieces small enough to place into mouse ovaries, whilst still being large enough to allow cell seeding. Therefore, a larger animal, such as the cow or, perhaps more accessible, sheep, would be necessary. To our knowledge, all studies reporting the use of Gelfoam® as a cell delivery strategy have used *in vivo* approaches to date: bone marrow MSC/Gelfoam® constructs did produce cartilage-like ECM *in vitro*, however, cell delivery to tissue was only performed *in vivo* (Ponticiello *et al.*, 2000). The "sandwich" structure used in our experiments was devised in an attempt to (a) expose as much of the surface area of the Gelfoam® to ovarian cortex as possible and (b) apply light pressure on the Gelfoam® to encourage absorption, or liquefaction. The cut edge of the ovarian cortex was chosen to be located next to the Gelfoam®, as it was hypothesised that this would more

likely enable invasion of the putative OSCs than the intact OSE. Furthermore, the innermost cortex/medullary region would be the intended location of the scaffold *in vivo* if it was to be used clinically. The "sandwich" was orientated so that the acupuncture needle was vertical, so that any effect of gravity on the putative bovine OSCs led to the cells coming into contact with cortex and not the culture medium.

It was only possible to culture the structures for 1 week: for ease of handling, the cortical pieces were larger than would normally be cultured *in vitro* (Telfer *et al.*, 2008, McLaughlin and Telfer, 2010) and follicular health was noted to be poor after this time period, indicating that *in vitro* culture was not fully maintaining the viability of the tissue. However, the freeze-thaw process may have contributed to this detrimental effect on follicle viability. It may be that this short culture period, and the lack of a vascular surface, was the reason why the Gelfoam® did not liquefy or was not absorbed by the tissue.

With regards the biomaterial used in these experiments, several different materials have been used as scaffolds to create artificial ovaries to date, as discussed in section 7.1. Gelatin, the principal constituent of Gelfoam®, has already been utilised successfully in the form of a 3D printed, porous, cross-linked structure, with livebirths produced when the structure, seeded with isolated murine follicles and ovarian cells, was autografted into recipient mice (Laronda *et al.*, 2016). The authors postulate that this structure, which is similar in characteristics to that of Gelfoam®, is important to optimise handling and allow follicle growth, ovulation and vascularisation of the scaffold (Laronda *et al.*, 2016). Indeed, no angiogenic factors were required in Laronda *et al.*'s study for live pups to be produced, in contrast to fibrin, which has been reported to require the addition of VEGF in order to achieve livebirths (Kniazeva *et al.*, 2015, Laronda *et al.*, 2016). This recent study therefore supports the use of gelatin-based scaffolds, such as Gelfoam®, for the purpose of cell delivery and folliculogenesis.

In contrast to the *in vitro* approach used in experiments detailed in this Chapter, the artificial ovary research reporting follicular growth and livebirths has thus far involved *in vivo* approaches (Luyckx *et al.*, 2014, Vanacker *et al.*, 2014, Kniazeva *et al.*, 2015, Laronda *et al.*, 2015, Chiti *et al.*, 2016, Laronda *et al.*, 2016). Furthermore, the

scaffolds were seeded with both follicles and additional somatic cells, thus providing the theca and stromal cells required for structural and endocrine support of the developing follicle. Thus, important further investigations would involve 1) auto- or xenografting of putative OSC-seeded Gelfoam® into sterilised or oophorectomised cows or sheep, with subsequent analysis for evidence of *neo*-folliculogenesis, and 2) seeding of Gelfoam® with both putative OSCs and ovarian somatic cells of the same species, to assess whether this allows new follicles to form within the scaffold itself.

7.4.2 Summary

Overall, Gelfoam® has shown promise as a scaffold for the retention of putative bovine OSCs; however, while rhodamine-labelled putative bovine OSCs could be identified within the Gelfoam®, including at the interface between the cortex and scaffold, no labelled cells were detected in the cortex. It has thus not been shown that Gelfoam® can efficiently deliver putative OSCs into tissue. Yet, these are preliminary results: further optimisation of the cell delivery strategy is required to assess whether it can efficiently deliver cells into the ovarian cortex. Future experiments should focus on *in vivo* strategies, as exposure to the physiological ovarian environment and vascular surfaces may be critical to improving cell delivery, and on co-seeding with ovarian somatic cells to ascertain whether the scaffold can support *neo*-folliculogenesis. Lastly, trialing of other types of cell scaffolds may also be of value, given the success of biomaterials such as fibrin, alginate and decellularised ovaries in supporting follicular and ovarian cell growth.

Chapter 8

General Discussion

8.1 Introduction

The overall aim of the research detailed in this thesis was to investigate the existence of OSCs within adult bovine and human ovarian cortex. This was, and indeed remains, a very novel and controversial field as little research into these cells existed at the time of commencing this body of work: indeed, only four groups had reportedly isolated the cells in only two species, mice and humans (Zou *et al.*, 2009, Pacchiarotti *et al.*, 2010, Zou *et al.*, 2011, Hu *et al.*, 2012, White *et al.*, 2012). Since then, OSCs have been reported in rats, pigs and rhesus macaque monkeys (Wolff *et al.*, 2013, Bui *et al.*, 2014, Wolff *et al.*, 2014, Zhou *et al.*, 2014, Hernandez *et al.*, 2015, Wolff, 2016); however, this thesis is the first to describe putative OSCs in the cow and substantiates only two previous reports of human OSCs (White *et al.*, 2012, Hernandez *et al.*, 2015). The experiments undertaken thus add to the growing body of evidence that has demonstrated a population of cells exists within adult mammalian ovaries which exhibit features of both stem cells and germ cells (Zou *et al.*, 2009, Zou *et al.*, 2011, Pacchiarotti *et al.*, 2010, Hu *et al.*, 2012, White *et al.*, 2012, Wolff *et al.*, 2013, Bui *et al.*, 2014, Wolff *et al.*, 2014, Zhou *et al.*, 2014, Hernandez *et al.*, 2015, Xiong *et al.*, 2015, Lu *et al.*, 2016, Wolff, 2016).

8.1.1 Putative OSC isolation

The investigations described in Chapter 3 demonstrated that a small number of DDX4-positive cells could be isolated from both species by a previously reported methodological approach (White *et al.*, 2012, Woods and Tilly, 2013). These cells, which were demonstrated by molecular characterisation to be distinct from oocytes, could be classified into two groups by cell size, with the smaller cells (Population A) being greater in number than the larger cells (Population B). The existence of two populations has not been described in the literature previously, except in pigs, where unsorted ovarian cells were analysed. However, given that other groups have reported ranges in cell size (Zou *et al.*, 2009, Pacchiarotti *et al.*, 2010, Zou *et al.*, 2011, White *et al.*, 2012), it may be that more than one cell population was present in these studies, but not detected. Examination of all the literature published to date on OSCs derived from adult ovarian cortex reveals differences, as well as similarities, between cell prevalence and non-molecular characteristics (Table 8.1). This supports the hypothesis that heterogeneous populations of cells are being isolated, both between

different groups of investigators as well as probably within different experimental approaches.

Heterogeneity of the cell type, coupled with methodological variations, may explain why some investigators (Zhang *et al.*, 2015, Zarate-Garcia *et al.*, 2016) have been unable to isolate or culture these cells, despite using the same isolation methodology as White *et al.* (White *et al.*, 2012): perhaps they are not isolating the same population as those who have reported cells with *neo*-oogenesis capabilities. A report on a collaboration between two groups utilising FACS to study human breast cells describes how, after meticulous investigation into the inability of one group to reproduce the other's data, it was discovered that the critical methodological variation accounting for the lack of reproducibility was the speed and length of agitation for tissue dissociation (Hines *et al.*, 2014). Similarly, it took our group several months to optimise White *et al*.'s isolation technique with small changes in tissue dissociation and gating strategies essential for successful detection of the desired cell population. Therefore, even seemingly small alterations in technique may have a great impact on the findings. Furthermore, recent unpublished work from Prof. Telfer's group suggests that careful examination of human DDX4-positive cells, isolated with the modified disaggregation protocol, reveals three discrete populations, of differing cell sizes and levels of DDX4 expression. Work is ongoing to elucidate the differences between the populations; however, it appears that one of the ways in which the populations can be differentiated is by their expression of different splice variants of *DDX4*. Analysis in several species including buffalo (*Bubalus bubalis*; Kaushik *et al.*, 2015), cattle (*Bos taurus*; Luo *et al.*, 2013), the tammar wallaby (a marsupial) and the platypus (Hickford *et al.*, 2011) has previously revealed that several splice variants of *DDX4* mRNA exist, some of which appear to be conserved in the mouse, at least *in silico* (Hickford et al., 2011). If OSCs express such splice variants, it may account for the lack of *DDX4* expression reported by some groups (Hernandez *et al.*, 2015, Zhang *et al.*, 2015, Zarate-Garcia *et al.*, 2016), as the primers utilised by the authors may not be specific to the *DDX4* variant expressed by the cells.

Table 8.1. Summary table of the findings of all the published reports of adult OSCs, with regards species, sorting technique, cell prevalence and cell description, allowing comparison with the data presented in this thesis.

Group	Species	Sorting Method	Marker	Cell size (µm)	Cell Prevalence	Morphology
Zou *et al.*, 2009	Mouse	MACS	DDX4	12 - 20	"50-100 cells from 6-8 adult mice"	Round
Pacchiarotti *et al.*, 2010	Mouse	FACS	POU5F1	10 - 15	0.05%	Round
Zou *et al.*, 2011	Mouse	MACS	IFITM3	12 - 20	Not stated from adult mice	Round
Hu *et al.*, 2012	Mouse	None	None	Not stated	Not stated	Elongated, spindle-like
White *et al.*, 2012	Mouse	FACS	DDX4	Not stated	1.5% ± 0.2%	Not described, but appear round in images
	Human	FACS	DDX4	5-8	1.7% ± 0.6%	Not described, but appear round in images
Wolff *et al.*, 2013, 2014, 2016	Macaque monkey	FACS	DDX4	Not stated	Not stated	Not described

Zhou *et al.*, 2014	Rat	MACS	IFITM3	Not stated	"200 – 300 cells from 20 ovaries"	Round
Bui *et al.*, 2014	Pig	None	None	5 – 7 and 10 - 12	Not stated	Round
Hernandez *et al.*, 2015	Mouse	FACS	DDX4	Not stated	1.9 – 3.7%	Not described
	Macaque monkey	FACS	DDX4	Not stated	2.5 – 50.6%	Not described
	Human	FACS	DDX4	Not stated	4.5 – 24%	Not described, but appear elongated in image
Xiong *et al.*, 2015	Mouse	MACS	IFITM3	Not stated	2%	Round
Lu *et al.*, 2016	Mouse	MACS	IFITM3	Not stated	5%	Round
This Thesis	Cow	FACS	DDX4	14 (Pop. A) 16 (Pop. B)	3.8% ± 1.2% (Pop. A) 1.0% ± 0.2% (Pop. B)	Elongated, spindle-like
	Human	FACS	DDX4	Not measured	1.9 ± 0.6% (Pop. A) 1.4 ± 0.7% (Pop. B)	Elongated, spindle-like

Another methodological aspect that could account for differences in results is the use of a polyclonal anti-DDX4 antibody. The controversy surrounding the use of DDX4 for OSC isolation has already been discussed in Chapter 1, with the specificity of the antibody used by our group being questioned subsequent to its apparently successful use in the experiments in this thesis (Hernandez *et al.*, 2015, Zhang *et al.*, 2015, Zarate-Garcia *et al.*, 2016). Perhaps less contentiously, isolation has also been performed using a more specific monoclonal anti-DDX4 antibody (Fakih *et al.*, 2015) and an alternate marker, IFITM3, which is known to contain an external epitope (Zou *et al.*, 2011, Zhou *et al.*, 2014, Xiong *et al.*, 2015, Lu *et al.*, 2016). However, recently, there have been calls to abandon the use of polyclonal and even current monoclonal antibodies in favour of standardised, sequenced recombinant antibodies due to the inherit variabilities of the former, resulting in difficulties with experimental reproducibility (Bradbury and Pluckthun, 2015). Furthermore, questions have been raised very recently regarding the use of IFITM3, as it has been reported to be more widely expressed within the mouse ovary than previously thought, indicating it is may be not be germ cell-specific (Zarate-Garcia *et al.*, 2016). An alternative, less controversial, marker for cell sorting is now necessary if one of the criticisms regarding the existence of OSCs is to be answered. C-KIT is a cell-surface receptor and thus a potential candidate (Saiti and Lacham-Kaplan, 2007) as it is expressed by human primordial germ cells both during migration to the gonadal ridge and during proliferation thereafter (Robinson *et al.*, 2001, Hoyer *et al.*, 2005). However its lack of germ cell specificity (Besmer *et al.*, 1993) means it is not an ideal choice. Moreover, the putative bovine OSCs only acquired detectable levels of *C-KIT* during *in vitro* culture. Detailed molecular characterisation of OSCs may provide an alternative option.

8.1.2 Putative OSC characterisation

Chapter 4 described the molecular signature of the isolated cells. Bovine and human cells exhibited molecular features of both stem cells and germ cells at the mRNA and protein level, indicating that they were putative OSCs. The characterisation described is one of the most comprehensive in the published literature and, in the case of the bovine experiments, included analysis of both freshly isolated and cultured cells, which has rarely been reported (White *et al.*, 2012, Hernandez *et al.*, 2015). This analysis provided confirmation that DDX4 was present in the freshly isolated cells at

both the mRNA and protein level. The overall molecular signature generally corroborated the findings of previous OSC research (Tables 8.2 and 8.3), with the exception of *NANOG*, *C-KIT* (both not detected by Wu's group in rodents (Zou *et al.*, 2009, Zhou *et al.*, 2014)), *DPPA3* (not detected in this research in bovine cells) and *DDX4* (not detected by Hernandez *et al*. (Hernandez *et al.*, 2015) and inconsistently detected in our experiments). Although lack of expression of these markers may be genuine findings, it has been suggested that failure to detect mRNA expression may be due to technical issues (Woods and Tilly, 2013). Certainly, Chapter 4 describes difficulties in detecting *DDX4*/DDX4, with alterations in isolation and molecular detection techniques required before expression could be confirmed.

Table 8.2. Summary table comparing the mRNA characterisation of putative OSCs in this thesis with that of the findings of all other published reports of adult OSCs. Grey boxes indicate that the marker was not analysed by the named group. * Detected in human cells only. ** Exhibited variable expression in both species (*DDX4*) or bovine cells only (*C-KIT*).

Marker (mRNA)	Group										
	Zou *et al.* (2009 and 2011)	Pacchiarotti *et al.* (2010)	Hu *et al.* (2012)	White *et al.* (2012)	Wolff *et al.* (2013 and 2014)	Zhou *et al.* (2014)	Bui *et al.* (2014)	Hernandez *et al.* (2015)	Xiong *et al.* (2015)	Lu *et al.* (2016)	This Thesis
POU5F1	✓	✓	✓			✓	✓				✓
LIN28											✓
NANOG	X		✓		✓	X	✓				✓ *
PRDM1	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
DPPA3	✓			✓	✓			✓			✓ *
IFITM3	✓			✓	✓	✓	✓	✓	✓	✓	✓
C-KIT	X	✓				X	✓				✓ **
DDX4	✓	✓		✓		✓	✓	X	✓	✓	✓ **

Table 8.3. Summary table comparing the protein characterisation of putative OSCs in this thesis with that of the findings of all other published reports of adult OSCs. Grey boxes indicate that the marker was not analysed by the named group. * Detected in bovine cells only. ** Detected in human cells only.

Marker (Protein)	Group										
	Zou *et al.* (2009 and 2011)	Pacchiarotti *et al.* (2010)	Hu *et al.* (2012)	White *et al.* (2012)	Wolff *et al.* (2013 and 2014)	Zhou *et al.* (2014)	Bui *et al.* (2014)	Hernandez *et al.* (2015)	Xiong *et al.* (2015)	Lu *et al.* (2016)	This Thesis
POU5F1	✓	✓	✓			✓	✓				✓ *
LIN28			✓								✓
NANOG	X	✓	✓								
PRDM1	✓			✓			✓				
DPPA3	✓			✓			✓				
IFITM3	✓			✓			✓		✓	✓	✓ **
C-KIT		✓					✓				✓
DDX4	✓		✓				✓	X	✓	✓	✓
DAZL											✓

Differences in OSC marker detection between studies may also be a result of *in vitro* transformation. Characterisation of putative bovine and human OSCs demonstrated that this phenomenon might be occurring, with acquisition of *C-KIT* expression (bovine cells) and variable expression of *DDX4* (both species). Therefore, the time points at which cells were analysed by other groups may have contributed to failure in the detection of some markers. Moreover, murine research that recently provided evidence of a physiological role for OSCs, has reported the *in vivo* transformation that the cells undergo: a small number of Pou5f1-positive, mitotic "germ stem cells" exhibited successive loss of Pou5F1, acquisition of Dazl and finally acquisition of Ddx4, and apparently entered meiosis, with Stra8 and Sycp3 expression detected (Guo *et al.*, 2016; Fig. 8.1). This was accompanied by an increase in cell size.

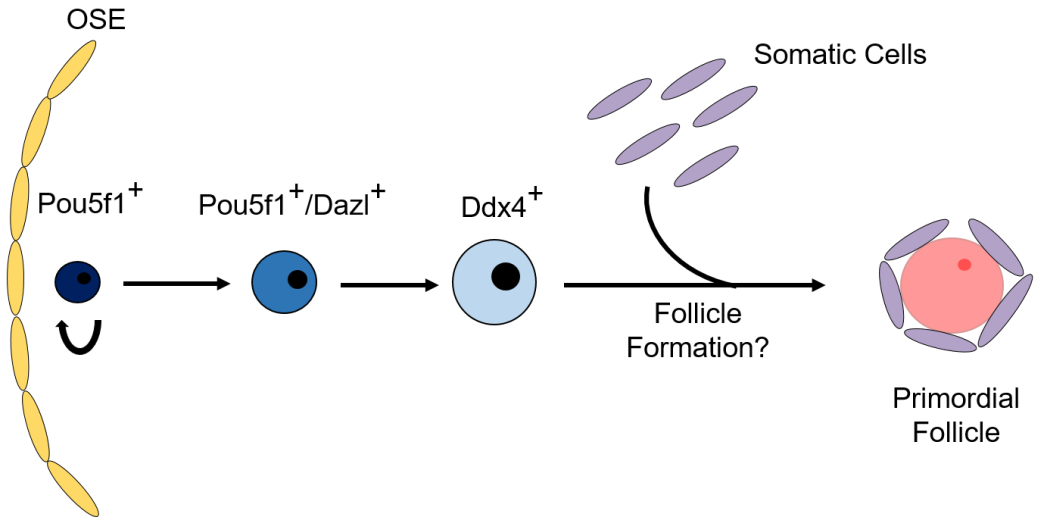


Figure 8.1. Schematic of the results reported by Guo *et al.* (Guo *et al.*, 2016). A population of Pou5f1-positive "germ stem cells" was discovered at the ovarian surface epithelium (OSE) of juvenile mouse ovaries, which appeared to proliferate. Some of the daughter cells were reported to enter a developmental pathway, with successive acquisition of Dazl and Ddx4 and loss of Pou5f1, during which time the cells increased in size. These cells were purported to initiate meiosis and subsequently undergo follicle formation, with replenishment of the ovarian reserve. Adapted from Anderson and Telfer, 2016.

This developmental pathway has similarities to that seen in PGCs within the mammalian fetal ovary. For example, in mice, *Pou5f1* expression in PGCs precedes and overlaps with *Dazl* and *Ddx4* expression, with pre-migratory and migratory PGCs expressing *Pou5f1* but lacking *Dazl* and *Ddx4* (Saiti and Lacham-Kaplan, 2007). It is only after migration to the genital ridges has been completed that PGCs begin to express *Dazl* and *Ddx4* and *Pou5f1* expression starts to decline (Saiti and Lacham-Kaplan, 2007).

This research not only provides new evidence that OSCs may have a physiological role in replenishing the ovarian reserve, at least in mice, it also offers a possible explanation for the heterogeneity of the reported OSCs, both in terms of cell size and molecular characterisation. For example, OSCs isolated by Pou5f1 expression (Pacchiarotti *et al.*, 2010) were slightly smaller than some cells isolated by Ddx4/DDX4 (Zou *et al.*, 2009, Zou *et al.*, 2011, and results from this thesis), which would fit with Guo *et al*.'s findings (Guo *et al.*, 2016). Furthermore, it may be that isolation of putative OSCs by DDX4 is targeting a population of cells further along the developmental programme and thus they may have lost, or gained, expression of markers compared with cells earlier in the pathway, and their characteristics may change further in culture. They also may have lost some of their stem cell features, such as immortal self-renewal.

In addition to *in vitro* transformation, there was evidence in our research that spontaneous *in vitro* differentiation into oocytes may be occurring, with acquisition of mRNA of oocyte-specific markers (*NOBOX* in bovine cells and *GDF-9* in human cells) and detection, rarely, of OLCs as assessed by morphology. Yet, characterisation did not verify that they were genuine oocytes and therefore expression of oocyte-specific markers was likely occurring in the adherent cells, rather than the non-adherent OLCs. However, acquisition of oocyte markers is not proof that oogenesis is occurring, therefore the functional capabilities of the cells necessitated investigation.

8.1.3 The functional capabilities of putative OSCs

The *in vitro* potential of putative bovine and human OSCs was examined in Chapters 5 and 6. In Chapter 5, the incorporation of the cells into two distinct *in vitro* culture systems was explored with the aim of observing *neo*-oogenesis. Injection of putative bovine and human OSCs into adult ovarian cortex and re-aggregation of the cells with fetal ovarian somatic cells was performed, with subsequent histological analysis. Although human cells were observed, rarely, in close association with somatic cells, with some in primordial follicle-like structures, no definite proof of *neo*-oogenesis was evident. The published literature on OSCs has described differing functional endpoints with regards oocyte formation. Table 8.4 places the experimental findings of this research in the context of the wider OSC literature. *In vitro* approaches have evidently not been as successful as *in vivo* strategies, with no development past the formation of OLCs and follicle-like structures.

Table 8.4. Summary table comparing the oogenesis potential of putative OSCs in this thesis with that of the findings of all other published reports of adult OSCs.

Group	Species	Culture Strategy	Functional endpoint
Zou *et al*., 2009	Mouse	*In vivo*	Live offspring
Pacchiarotti *et al*., 2010	Mouse	*In vitro*	OLCS and follicle-like structures
Zou *et al*., 2011	Mouse	*In vivo*	Not stated
Hu *et al*., 2012	Mouse	*In vitro*	OLCs
White *et al*., 2012	Mouse	*In vivo*	Hatching blastocysts
	Human	*In vivo*	Immature follicles
Wolff *et al*., 2013 and 2014	Macaque monkey	*In vivo*	Mature oocytes
Wolff, 2016	Macaque monkey	*In vivo*	Embryos
Zhou *et al*., 2014	Rat	*In vivo*	Live offspring
Bui *et al*., 2014	Pig	*In vitro*	OLCs
Hernandez *et al*., 2015	Mouse	*In vitro*	OLCs by morphology only
	Macaque monkey	*In vitro*	OLCs by morphology only
	Human	*In vitro*	OLCs by morphology only
Xiong *et al*., 2015	Mouse	*In vivo*	Live offspring
Lu *et al*., 2016	Mouse	*In vivo*	Live offspring
This Thesis	Cow	*In vitro*	OLCs by morphology only
	Human	*In vitro*	OLCs and follicle-like structures by morphology only

Given that many aspects of PGC development remain unexplored, it is reasonable to assume that the *in vivo* milieu better provides for the requirements of OSCs than the

in vitro environment. As such, *in vivo* approaches for putative bovine OSC culture are currently being undertaken by a fellow PhD student in Prof. Anderson's and Prof. Telfer's groups in order to examine their oogenesis potential further. The use of Gelfoam® as a cell scaffold, as described in Chapter 7, may provide a novel *in vivo* strategy for the investigation of OSC potential.

In Chapter 6, the potential of putative bovine and human OSCs as a germ cell model was investigated, by analysing their response to two key regulators of PGC development, BMP4 and RA. Despite the lack of conclusive evidence that *neo*-oogenesis was occurring *in vitro*, the investigations indicated that bovine and human cells were behaving as *bona fide* OSCs would be expected to in response to these regulators. The molecular responses mimicked those of PGCs, with evidence of upregulation of a critical meiotic entry gene, *SYCP3*, in response to BMP4 and RA. This was similar to the findings of the only other published report on OSCs and BMP4 treatment, which utilised mouse OSCs (Park *et al.*, 2013). These were preliminary experiments to explore the potential of the cells to act as germ cell models; however, the findings suggested that the bovine cells at least, may be a promising *in vitro* approach to exploring the intricate complexities of the PGC developmental programme.

8.2 Novel aspects of the research

The principle novel finding of the research detailed in this thesis was the discovery of putative OSCs in the cow. Although OSCs have now been reported in pigs (Bui *et al.*, 2014) and rhesus macaque monkeys (Wolff *et al.*, 2013, Wolff *et al.*, 2014, Wolff, 2016), at the time of the isolation and initial characterisation of the bovine cells, OSCs had not been reported in any large animal model. As already discussed in Chapter 3, the similarities between bovine and human follicular development have led to a large body of work exploring the cow as an important model for human ovarian development, and as such, with the exception of monkey OSCs, bovine OSCs are probably the most valuable type of OSCs yet reported, with respect to translation to humans. An additional novel aspect, with regards cell isolation, was the isolation of two different DDX4-positive populations from adult ovarian cortex. Two discrete populations of OSCs have been described previously in pigs; however, a purified, sorted population was not analysed in this study (Bui *et al.*, 2014). Although not novel,

the isolation of putative human OSCs had only been reported once when the research contained within this thesis began (White *et al.*, 2012) and was not confirmed by others until recently (Hernandez *et al.*, 2015). Even then, Hernandez *et al*.'s findings (Hernandez *et al.*, 2015) were not entirely consistent with White *et al.*'s results (White *et al.*, 2012), with the former group unable to demonstrate DDX4 expression at either the mRNA or protein level. The research in this thesis provides data in the middle ground of these two papers: *DDX4*/DDX4 expression could be detected in the cells isolated in this thesis, however, its expression was not consistent. As discussed in section 1.2.5.3, reproducibility of data is critical in science and, therefore, although our human findings were not novel, they still demonstrate an important development in the OSC field.

With respect to another aspect of cell characterisation, Western blotting was utilised for the first time to our knowledge to demonstrate expression of germline markers, providing additional confirmation of the ICC findings that the cells express these proteins. DDX4 analysis of OSCs by immunoblotting has been reported once before; however, no protein was detected in that study (Hernandez *et al.*, 2015). A feature of the research that was novel at the time of the experiments was the use of lentiviruses for transduction. Lentiviral transduction of OSCs for the purposes of fluorescent labelling has since been published by other groups (Wolff *et al.*, 2013, Wolff *et al.*, 2014, Xiong *et al.*, 2015, Lu *et al.*, 2016), although none of these studies reported a detrimental effect on cell viability.

During the investigation of the *in vitro* potential of the putative bovine and human OSCs, two novel approaches to *in vitro* OSC culture were devised, by adapting cortical injection and re-aggregated ovary strategies that had been shown to be successful *in vivo* (Eppig and Wigglesworth, 2000, White *et al.*, 2012). Moreover, for the first time, RA-responsiveness and the effect of overexpression of a gene related to meiosis (i.e. *DAZL*) on putative OSCs was investigated. Finally, a novel OSC delivery system, using a Gelfoam® cell scaffold, was explored: future studies regarding this preliminary study will be discussed in section 8.5.

8.3 Limitations of the research

Clearly, the fact that our investigations have not been able to prove definitively the ability of the isolated cells to generate new oocytes is the principal limitation of the

research presented within this thesis, and means that the cells can only be termed putative OSCs. The cells could perhaps be another type of ovarian stem cell that is permissive and can be reprogrammed to behave like an OSC under the right *in vitro* conditions, although freshly isolated putative bovine OSCs also expressed stem cell and germline markers, supporting the hypothesis that the isolated cells were OSCs. However, evidence suggestive of *in vitro* transformation is a limitation to the use of the cells in clinical and basic science applications as it indicates that the long-term expansion of the cells may not be possible without an effect on germline function. This could have an impact on their clinical use, as freshly isolated cells may have to be utilised for the generation of new oocytes, which will greatly restrict the number of cells available to a patient. It also affects their use as a germ cell model as *in vitro* transformation may result in a heterogeneous population with differing responses to signals which do not mimic that of germ cells. Additionally, with regards clinical use, the retrieval of cells may not be possible in all women as our studies showed that the isolated cells of three of the eight women did not establish in culture. Although that may reflect the limitations of the current isolation and culture methods, as low numbers of cells were isolated in these experiments due to high levels of cell death, it may be that OSCs are not a viable fertility preservation option for all women.

In terms of the germ cell model experiments described in Chapter 6, the induction of genes involved in meiotic initiation by BMP4 and RA treatment was detected; however, the studies met none of the standards proposed by Handel *et al*. for the evaluation of meiosis in *in vitro*-derived germ cells (Handel *et al.*, 2014). The authors recommended that in order to confirm *in vitro* oogenesis, the following benchmarks had to be met:

1) Verification of DNA content from the pre-meiotic stage until after the second meiotic division, with the correct content observed at each critical stage.
2) Evaluation of chromosome counts and correct organisation on the spindle, particularly in MII oocytes and early embryos.
3) Evidence of chromosomal recombination.
4) Ultimately, generation of live, euploid offspring (Handel *et al.*, 2014).

Although ICC demonstrated the expression of SYCP3 protein within the nuclei of the treated cells, chromosomal spreads were not performed to assess chromosomal recombination. Therefore, the results presented in this thesis appear to indicate

induction of meiosis, or at least some aspects of it, but have not provided gold standard proof.

Many of the experiments described would have benefitted from a greater number of replicates to increase reliability; however, tissue and cell availability and time constraints were limiting factors in this regard. Bovine tissue is more freely available than human tissue, yet, it is still limited in supply, preventing more large-scale investigations. This was especially true in the studies characterising freshly isolated cells: this could not be performed in humans due to lack of tissue and was limited in bovine due to the amount of tissue required per experiment. Due to time limitations, several investigations could not be pursued further, including isolation of cells using IFITM3 as an alternative marker, optimisation of the *in vitro* culture system for aggregated "ovaries", further exploration of meiotic entry in BMP4 and RA treated cells and development of the Gelfoam® cell scaffold studies. With regards the aggregated "ovary" experiments, it may be that longer *in vitro* culture periods are required for confirmed *neo*-oogenesis to occur; however, given the extent of cell death detected within 1 – 2 weeks of culture, improvements in the culture system are imperative to support cell viability for more extended time periods. Future work, discussed in section 8.5, will hopefully address many of the issues discussed in this section.

8.4 Applications of OSCs

Despite the controversies, the discovery of putative OSCs has elicited much discussion and interest amongst reproductive biologists, as not only has it led to the questioning of a longstanding dogma, but the applications of the cells, in basic science, agricultural and clinical fields are potentially cutting-edge.

8.4.1 Basic science applications

In addition to their putative role as an *in vitro* model for germ cell development, discussed in Chapter 6, OSCs are already being utilised to create transgenic rodents (Zhang *et al.*, 2011, Zhou *et al.*, 2014). The use of transgenic animal models is a well-established, valuable tool in basic science to allow investigation of gene function. Several different techniques for generating transgenic animals, with varying degrees of efficiency and cost, have been developed over the last few decades, including, but not restricted to, microinjection of DNA fragments into the pronucleus of a one-cell

embryo, use of viral vectors to introduce desired DNA into a nucleus and sophisticated, inducible Cre-LoxP systems which integrate DNA into a cell's genome and can cause conditional gene knock-outs (Houdebine, 2007). OSCs provide an effective, novel technique of generating transgenic animals with putative murine OSCs already reported to produce transgenic mice and rats, via a rapid and inexpensive approach (Zhang *et al.*, 2011, Zhou *et al.*, 2014). Three methods have been utilised thus far: retrovirus transduction, lentivirus transduction and liposome-mediated transfection (Zhang *et al.*, 2011, Zhou *et al.*, 2014).

Recombinant retroviruses and lentiviruses have been utilised to create transgenic GFP-expressing OSCs by several groups and, in some cases, when transplanted into sterile mice which were mated with wild-type males, these OSCs have generated heterozygous GFP-expressing offspring which can pass their transgene onto the next generation (Zou *et al.*, 2009, Zhang *et al.*, 2011, White *et al.*, 2012, Wolff *et al.*, 2013, Wolff *et al.*, 2014, Wolff, 2016). However, retrovirus-mediated transduction of OSCs has also generated transgenic mice which can overexpress the *Oocyte-G1* and *dynein axonemal intermediate chain 2* (*Dnaic2*) genes with high efficiency (Zhang *et al.*, 2011). The function of these genes is poorly understood, therefore analysis of the transgenic mice allowed possible roles to be hypothesised, with mice overexpressing *Dnaic2* demonstrating a subfertile (female) or sterile (male) phenotype. In the same study, liposomal transfection was performed to create knockdown mice by introducing a short hairpin RNA (shRNA) into the OSC genome to "silence" the expression of *Oocyte-G1*. Again, analysis of the transgenic offspring allowed theories regarding the function of the gene to be postulated, with mice demonstrating gonadal and brain abnormalities (Zhang *et al.*, 2011).

Rats OSCs have also been genetically manipulated to produce transgenic offspring in an efficient manner. Liposome-mediated transfection resulted in expression of both GFP and the *fat-1* gene and in the case of *fat-1*, after transplantation into infertile rats and mating with wild-type males, 28% of the offspring were heterozygous for the gene (Zhou *et al.*, 2014). These offspring were generated in 2 months, were healthy and demonstrated the phenotype expected of a *fat-1* overexpressing animal (i.e. higher levels of n-3 fatty acids compared to wild-type mice). Thus, OSCs have already been demonstrated to be an efficient approach to producing transgenic animals which could prove extremely useful in basic science research.

Finally, Wu's group have demonstrated that pre-pubertal mouse OSCs can be dedifferentiated into embryonic stem-like cells (ESLCs) under certain *in vitro* culture conditions (Wang *et al.*, 2014). ESLCs were shown to be pluripotent during *in vitro* characterisation and they formed teratomas *in vivo*, unlike OSCs. When GFP-expressing ESLCs were injected into wild-type blastocysts, the resultant fetuses contained GFP-positive cells in organs derived from all three germ layers, including heart, muscle and testes. Such findings will be important in elucidating further the processes involved in *in vitro* stem cell reprogramming and, indeed, may have potential in regenerative medicine applications (Wang *et al.*, 2014).

8.4.2 Agricultural applications

Cattle are an extremely valuable species in agriculture for meat and dairy production: the Department for Environment, Food and Rural Affairs (Defra) reported that the cattle population in the UK in 2015 rose to 9.9 million, with an economic value of £2,739 million (Defra, 2016). Globally, bovine embryos are produced *in vitro* in large quantities in order to enhance breeding for desired characteristics (Hall *et al.*, 2013). Bovine IVF allows the embryos of genetically valuable heifers and bulls to be implanted into several less valuable surrogate heifers and can result in a prized heifer producing many offspring in one year. Cloning is also a well-established technique by which genetically modified cattle can be produced in order that preferred genetic characteristics are propagated (Cibelli *et al.*, 1998). OSCs may have a valuable role in agricultural applications as cells from a genetically superior heifer could be used as a source of oocytes for bovine IVF. Secondly, transgenic bovine OSCs could be used to generate genetically modified cattle that may have both agricultural and basic science (see section 8.4.1) applications.

Another theoretical role for OSCs in both agriculture and the wider animal kingdom, could be in species conservation. Cloning has already been used to save an endangered species of cow (the Enderby Island cow; Wells *et al.*, 1998)) and a species of wild sheep (*Ovis orientalis musimon*; Loi *et al.*, 2001)) from extinction, and it is hoped it could even be used to resurrect extinct species, such as the mammoth (Stone, 1999). Again, OSCs from the ovaries of endangered (or indeed, preserved extinct) animals may be theoretically utilised to create oocytes or provide the genetic material required for nuclear transfer into the enucleated oocyte of a genetically similar animal.

8.4.3 Clinical applications

Currently, there are three possible fertility preservation options for post-pubertal girls and women who know they are at risk of POI: 1) oocyte cryopreservation after ovarian stimulation, 2) embryo cryopreservation after ovarian stimulation and *in vitro* fertilisation (IVF) with their partner's sperm, and 3) ovarian cortex cryopreservation with re-implantation of the thawed tissue after the patient is disease-free. The last approach is the only available strategy for pre-pubertal girls (Wallace *et al.*, 2005, Anderson and Wallace, 2011) and women who cannot afford the delay in life-saving treatment that ovarian stimulation would inevitably cause. Unfortunately, none of the current approaches result in a large number of oocytes being obtained for future use. In addition, although girls and women with recognised genetic causes of POI, such as Turner's syndrome, may present in time for fertility preservation strategies to be possible, these options are often not feasible for women who experience idiopathic POI as they present too late. In such cases, where there is approximately only a 5% chance of a spontaneous conception (Goswami and Conway, 2007), oocyte donation and IVF currently offer the best opportunity to conceive. Other drawbacks include delays in potentially life-saving treatment (if ovarian stimulation is required) and, in the case of ovarian cortex cryopreservation in cancer patients, the risk of reintroducing malignant cells during the re-implantation of the tissue, particularly in those with haematological malignancies (Dolmans *et al.*, 2010, Rosendahl *et al.*, 2010). OSCs may be able to circumvent these disadvantages and also be an additional option for pre-pubertal girls.

The theoretical technique would involve removing a sample of ovarian cortex prior to commencing infertility-inducing treatment, isolating the OSCs and then either culturing and maturing the cells *in vitro* to generate potentially large numbers of oocytes which could be used in IVF, or injecting the cells back into a woman's ovaries following disease remission. As discussed in Chapter 7, intra-ovarian OSC injection could potentially replenish the woman's ovarian reserve and restore endocrine function, at least on a temporary basis. The success of this approach might be constrained by the health of the stromal cells within the ovary: these cells, which would be critical for *neo*-folliculogenesis to occur, may have been negatively affected by the cancer treatment and therefore may not be able to support the differentiation and development of OSCs. However, if this approach worked, there would be no delay in commencing treatment, no risk of introducing malignant cells and the generation of

much larger numbers of oocytes than is currently possible. Additionally, as demonstrated in this thesis and by others (White *et al.*, 2012), the cells can be isolated from cryopreserved, as well as fresh tissue, meaning that cortex can be stored for a long time prior to OSC isolation, thus allowing advances in technologies to be occur. The finding that OSCs can be isolated from chemotherapy-exposed ovaries in mice, and used therapeutically to rescue infertility, also provides promise for those individuals who have already received cancer treatment (Xiong *et al.*, 2015). As such, there is no doubt that the potential of OSCs is deserving of further investigation.

A more speculative clinical use of OSCs would be in women suffering from age-related ovarian insufficiency, i.e. the menopause. The current secular trend for delaying childbearing in the developed world is resulting in some women having to resort to oocyte donation or undergoing pre-emptive "social" oocyte storage in order to have a child. Infertility is not the only aspect of concern, however, with the menopause associated with troublesome climacteric symptoms such as vasomotor effects (hot flushes, night sweats) and mood changes (Nelson, 2008). As discussed in section 1.2.4.1., OSCs have been reportedly detected in aged mouse ovaries (Niikura *et al.*, 2009), therefore, menopausal women hypothetically may possess a scarce population of OSCs that could be isolated from ovarian cortex biopsies. These cells could then be cultured *in vitro* to generate oocytes for use in IVF. The reversal of detrimental menopausal symptoms is perhaps more aspirational as it appears that the age of the surrounding stromal milieu is essential for OSCs to undergo oogenesis (Niikura *et al.*, 2009); therefore merely injecting OSCs back into an aged ovarian environment may not reinitiate ovarian function.

OSCs have already been used clinically in a slightly different way to aid women with age-related subfertility, using a procedure termed AUtologous Germline-derived Mitochondrial ENergy Transfer (AUGMENT) (Tilly and Sinclair, 2013). In this procedure, OSCs are isolated from a woman's ovarian biopsy and the mitochondria from these cells are purified before being transferred into the women's own oocytes. As it is believed that oocytes from older women may be of poorer quality due to decreased energy availability in the form of mitochondrial-derived adenosine triphosphate (ATP) (Bentov *et al.*, 2011), it is thought that transferring the purified OSC mitochondria into oocytes will "boost" their energy and enhance their ability to be fertilised and undergo healthy embryogenesis (Tilly and Sinclair, 2013). Indeed, it has been demonstrated that pig oocytes with reduced levels of mitochondrial DNA

(mtDNA) are less developmentally competent and mitochondrial supplementation (isolated from MII oocytes) of such oocytes significantly improves the number of oocytes reaching the blastocyst stage following fertilisation (Cagnone *et al.*, 2016). It is postulated that the OSCs will have accumulated less damage to their mitochondria with age as they are slowly dividing cells and is reported that the mitochondria of these cells have greater ATP production than ESCs and other adult somatic stem cells, therefore justifying their use (Tilly and Sinclair, 2013). Furthermore, it is an autologous treatment, therefore no DNA from other humans is required (thus avoiding the ethical difficulties of so-called "three parent babies") and as mtDNA is maternally inherited, this means all the offspring's mitochondria contain their mother's DNA. Babies have been reportedly born following this treatment (Couzin-Frankel, 2015), although evidence thus far is anecdotal, and robust randomised trials will be necessary to prove that this is as the direct result of the OSC mitochondria transfer.

In summary, the applied use of OSCs for oogeneic clinical purposes remains speculative, but the potential beneficial impact they could have in the field of oncofertility, and beyond, means that there is great value in investigating their capabilities further.

8.5 Future directions

Future work will be essential to analyse the cells' characteristics further and assess their oogenesis potential. Colleagues in Prof. Telfer's group have already modified the dissociation protocol to improve cell yields, as discussed in Chapters 3 and 4, which is allowing further elucidation of the *DDX4* expression of the freshly isolated cells. It may also enable the discovery of a novel, specific OSC marker which would be a significant breakthrough in the field, circumventing the controversies around DDX4 and possibly now IFITM3. Prof. Telfer's group is also now beginning to optimise the *in vitro* aggregated "ovary" experiments further with putative human OSCs. The *in vivo* potential of the cells from both species will be important to investigate and, as seen in the published work on OSCs (Table 8.4), may yield results more quickly than the *in vitro* approach. Work is already ongoing using the bovine cells within Prof. Anderson's laboratory, involving the xenografting of aggregated "ovaries" into nude mice. Hopefully, this will help determine whether the cells are genuine OSCs or not.

In order to determine whether the upregulation of meiosis entry-related genes reflects true initiation of meiosis, future investigations will have to assess at least three of the four standards set by Handel *et al*. (detailed in section 8.3; Handel *et al.*, 2014). DNA content of BMP4 and RA treated cells could be assessed by flow cytometry, whilst chromosomal counts and organisational changes could be assessed by FISH and chromosomal spreads with immunological detection of recombination-related proteins, e.g. DMC1. With regards the generation of viable offspring, fertilisation of bovine OSC-derived oocytes and intrauterine transfer of resultant embryos into a surrogate heifer would be feasible; however, legal and ethical barriers would currently prevent the investigation of the fertilisation capacity of human OSC-derived oocytes, with Human Fertilisation and Embryology Authority (HFEA) approval required for such experiments. Moreover, the production of healthy offspring is reliant on the correct imprinting of OSC-derived oocytes. Mouse *in vitro* follicle culture research has demonstrated evidence of correct imprinting in oocytes by DNA methylation (Anckaert *et al.*, 2009, Trapphoff *et al.*, 2010, El Hajj *et al.*, 2011), whilst one bovine oocyte IVM study (Heinzmann *et al.*, 2011) has also provided reassuring results. However, these studies look at oocytes that are already formed: differentiation of an OSC into an oocyte and subsequent IVM is likely a more complex process, perhaps with the potential for more epigenetic errors to occur. Combined bisulfite restriction analysis (COBRA) enables detection of DNA methylation of specific loci (Eads and Laird, 2002) and has been utilised by Wu's group to demonstrate that rodent OSCs were maternally imprinted, with partial methylation of maternally imprinted regions of DNA and demethylation of paternally imprinted regions detected (Zou *et al.*, 2009, Zhou *et al.*, 2014). These OSCs were then reported to produce viable offspring. Such a technique could also be utilised in bovine and human OSCs and OSC-derived oocytes.

Finally, the pilot studies utilising Gelfoam® as a cell scaffold could be modified for use *in vivo*: due to the structure of the material and the size of the ovaries, grafting of OSC-seeded Gelfoam® into rodent ovaries would be technically challenging, although grafting of the bovine "sandwiches" into nude mice may be productive. A larger animal model, such as the cow or sheep, would be preferable, however. This strategy would allow practice of the insertion technique of Gelfoam® into ovaries and be more translatable to clinical use in humans.

8.6 Concluding remarks

The research presented in this thesis demonstrates that, in support of previous research, putative OSCs can be isolated from adult human ovarian cortex and, for the first time, they have been extracted from adult bovine ovaries. A variety of approaches, including many novel strategies, were utilised to investigate the nature and potential of these cells and the findings have provided a strong foundation on which future work can be based. The investigations have revealed that the isolated cells bear the molecular signature of OSCs and mimic the response of PGCs to key regulators of the germ cell development pathway. Their oocyte-forming capabilities have not been proven; however, the optimal *in vitro* environment within which the putative OSCs may develop into germ cells is unknown. The work described within this thesis represents some of the first attempts at establishing favourable conditions for putative OSC differentiation, including investigation of support cells, matrices and differentiation-inducing factors. The concept of the OSC remains controversial in the field of reproductive biology; however, the data presented here show clearly that a population of cells exists within adult ovaries that have both stem cell and germline characteristics, although the quality and quantity of the isolated cells is dependent on the isolation methodology. Whether these cells have a physiological role within the ovary remains unclear. OSCs have great potential in basic science, agricultural and clinical applications, yet their use in these areas remains mostly aspirational at present. Much more research is required before such cells can be translated to the clinical setting in order to ensure the health and developmental competence of OSC-derived oocytes.

References

Abban G & Johnson J (2009). Stem cell support of oogenesis in the human. *Hum Reprod.* 24(12): 2974-8.

Abir R, Roizman P, Fisch B, Nitke S, Okon E, Orvieto R & Ben Rafael Z (1999). Pilot study of isolated early human follicles cultured in collagen gels for 24 hours. *Hum Reprod.* 14(5): 1299-301.

Aerts JM & Bols PE (2010). Ovarian follicular dynamics: a review with emphasis on the bovine species. Part I: Folliculogenesis and pre-antral follicle development. *Reprod Domest Anim.* 45(1): 171-9.

Aflatoonian B, Ruban L, Jones M, Aflatoonian R, Fazeli A & Moore HD (2009). In vitro post-meiotic germ cell development from human embryonic stem cells. *Hum Reprod.* 24(12): 3150-9.

Aitken RJ, Findlay JK, Hutt KJ & Kerr JB (2011). Apoptosis in the germ line. *Reproduction.* 141(2): 139-50.

Albertini DF & Gleicher N (2015). A detour in the quest for oogonial stem cells: methods matter. *Nat Med.* 21(10): 1126-7.

Allen E (1923). Ovogenesis during sexual maturity. *American Journal of Anatomy.* 31(5): 439-481.

Ambros V (2000). Control of developmental timing in Caenorhabditis elegans. *Curr Opin Genet Dev.* 10(4): 428-33.

Anckaert E, Adriaenssens T, Romero S, Dremier S & Smitz J (2009). Unaltered imprinting establishment of key imprinted genes in mouse oocytes after in vitro follicle culture under variable follicle-stimulating hormone exposure. *Int J Dev Biol.* 53(4): 541-8.

Anckaert E, De Rycke M & Smitz J (2013). Culture of oocytes and risk of imprinting defects. *Hum Reprod Update.* 19(1): 52-66.

Anderson R, Fassler R, Georges-Labouesse E, Hynes RO, Bader BL, Kreidberg JA, Schaible K, Heasman J & Wylie C (1999). Mouse primordial germ cells lacking beta1 integrins enter the germline but fail to migrate normally to the gonads. *Development.* 126(8): 1655-64.

Anderson RA, Fulton N, Cowan G, Coutts S & Saunders PT (2007). Conserved and divergent patterns of expression of DAZL, VASA and OCT4 in the germ cells of the human fetal ovary and testis. *BMC Dev Biol.* 7: 136.

Anderson RA & Telfer EE (2016). Replenishing the adult ovarian follicle population: a fresh look at dogma. *Mol Hum Reprod.* 22(5): 313-5.

Anderson RA & Wallace WH (2011). Fertility preservation in girls and young women. *Clin Endocrinol (Oxf).* 75(4): 409-19.

Antonio-Rubio NR, Porras-Gomez TJ & Moreno-Mendoza N (2013). Identification of cortical germ cells in adult ovaries from three phyllostomid bats: Artibeus jamaicensis, Glossophaga soricina and Sturnira lilium. *Reprod Fertil Dev.* 25(5): 825-36.

Bai Y, Yu M, Hu Y, Qiu P, Liu W, Zheng W, Peng S & Hua J (2013). Location and characterization of female germline stem cells (FGSCs) in juvenile porcine ovary. *Cell Prolif.* 46(5): 516-28.

Baillet A & Mandon-Pepin B (2012). Mammalian ovary differentiation - a focus on female meiosis. *Mol Cell Endocrinol.* 356(1-2): 13-23.

Bakhtari A & Ross PJ (2014). DPPA3 prevents cytosine hydroxymethylation of the maternal pronucleus and is required for normal development in bovine embryos. *Epigenetics.* 9(9): 1271-9.

Baltus AE, Menke DB, Hu YC, Goodheart ML, Carpenter AE, De Rooij DG & Page DC (2006). In germ cells of mouse embryonic ovaries, the decision to enter meiosis precedes premeiotic DNA replication. *Nat Genet.* 38(12): 1430-4.

Bartholomew RA & Parks JE (2007). Identification, localization, and sequencing of fetal bovine VASA homolog. *Anim Reprod Sci.* 101(3-4): 241-51.

Bath LE, Tydeman G, Critchley HO, Anderson RA, Baird DT & Wallace WH (2004). Spontaneous conception in a young woman who had ovarian cortical tissue cryopreserved before chemotherapy and radiotherapy for a Ewing's sarcoma of the pelvis: case report. *Hum Reprod.* 19(11): 2569-72.

Bayne RA, Kinnell HL, Coutts SM, He J, Childs AJ & Anderson RA (2015). GDF9 is transiently expressed in oocytes before follicle formation in the human fetal ovary and is regulated by a novel NOBOX transcript. *PLoS One.* 10(3): e0119819.

Beer RL & Draper BW (2013). nanos3 maintains germline stem cells and expression of the conserved germline stem cell gene nanos2 in the zebrafish ovary. *Dev Biol.* 374(2): 308-18.

Begum S, Papaioannou VE & Gosden RG (2008). The oocyte population is not renewed in transplanted or irradiated adult ovaries. *Hum Reprod.* 23(10): 2326-30.

Belli M, Vigone G, Merico V, Redi CA, Zuccotti M & Garagna S (2012). Towards a 3D culture of mouse ovarian follicles. *Int J Dev Biol.* 56(10-12): 931-7.

Bendsen E, Byskov AG, Andersen CY & Westergaard LG (2006). Number of germ cells and somatic cells in human fetal ovaries during the first weeks after sex differentiation. *Hum Reprod.* 21(1): 30-5.

Bentov Y, Yavorska T, Esfandiari N, Jurisicova A & Casper RF (2011). The contribution of mitochondrial function to reproductive aging. *J Assist Reprod Genet.* 28(9): 773-83.

Besmer P, Manova K, Duttlinger R, Huang EJ, Packer A, Gyssler C & Bachvarova RF (1993). The kit-ligand (steel factor) and its receptor c-kit/W: pleiotropic roles in gametogenesis and melanogenesis. *Development.* 119 Suppl: 125-37.

Bhartiya D, Sriraman K, Parte S & Patel H (2013). Ovarian stem cells: absence of evidence is not evidence of absence. *J Ovarian Res.* 6(1): 65.

Bortvin A, Goodheart M, Liao M & Page DC (2004). Dppa3 / Pgc7 / stella is a maternal factor and is not required for germ cell specification in mice. *BMC Dev Biol.* 4: 2.

Borum K (1967). Oogenesis in the mouse. A study of the origin of the mature ova. *Exp Cell Res.* 45(1): 39-47.

Bowles J, Knight D, Smith C, Wilhelm D, Richman J, Mamiya S, Yashiro K, Chawengsaksophak K, Wilson MJ, Rossant J, Hamada H & Koopman P (2006). Retinoid signaling determines germ cell fate in mice. *Science.* 312(5773): 596-600.

Bowles J & Koopman P (2007). Retinoic acid, meiosis and germ cell fate in mammals. *Development.* 134(19): 3401-11.

Bowles J, Teasdale RP, James K & Koopman P (2003). Dppa3 is a marker of pluripotency and has a human homologue that is expressed in germ cell tumours. *Cytogenet Genome Res.* 101(3-4): 261-5.

Bradbury A & Pluckthun A (2015). Reproducibility: Standardize antibodies used in research. *Nature.* 518(7537): 27-9.

Brinster RL (2007). Male germline stem cells: from mice to men. *Science.* 316(5823): 404-5.

Bristol-Gould SK, Kreeger PK, Selkirk CG, Kilen SM, Mayo KE, Shea LD & Woodruff TK (2006). Fate of the initial follicle pool: empirical and mathematical evidence supporting its sufficiency for adult fertility. *Dev Biol.* 298(1): 149-54.

Bui HT, Van Thuan N, Kwon DN, Choi YJ, Kang MH, Han JW, Kim T & Kim JH (2014). Identification and characterization of putative stem cells in the adult pig ovary. *Development.* 141(11): 2235-44.

Bukovsky A, Svetlikova M & Caudle MR (2005). Oogenesis in cultures derived from adult human ovaries. *Reprod Biol Endocrinol.* 3: 17.

Butler H & Juma MB (1970). Oogenesis in an adult prosimian. *Nature.* 226(5245): 552-3.

Byskov AG, Faddy MJ, Lemmen JG & Andersen CY (2005). Eggs forever? *Differentiation.* 73(9-10): 438-46.

Cagnone GL, Tsai TS, Makanji Y, Matthews P, Gould J, Bonkowski MS, Elgass KD, Wong AS, Wu LE, Mckenzie M, Sinclair DA & St John JC (2016). Restoration of normal embryogenesis by mitochondrial supplementation in pig oocytes exhibiting mitochondrial DNA deficiency. *Sci Rep.* 6: 23229.

Campbell BK, Souza C, Gong J, Webb R, Kendall N, Marsters P, Robinson G, Mitchell A, Telfer EE & Baird DT (2003). Domestic ruminants as models for the elucidation of the mechanisms controlling ovarian follicle development in humans. *Reprod Suppl.* 61: 429-43.

Castrillon DH, Miao L, Kollipara R, Horner JW & Depinho RA (2003). Suppression of ovarian follicle activation in mice by the transcription factor Foxo3a. *Science.* 301(5630): 215-8.

Castrillon DH, Quade BJ, Wang TY, Quigley C & Crum CP (2000). The human VASA gene is specifically expressed in the germ cell lineage. *Proc Natl Acad Sci U S A.* 97(17): 9585-90.

Chambers I, Colby D, Robertson M, Nichols J, Lee S, Tweedie S & Smith A (2003). Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell.* 113(5): 643-55.

Chang DH, Cattoretti G & Calame KL (2002). The dynamic expression pattern of B lymphocyte induced maturation protein-1 (Blimp-1) during mouse embryonic development. *Mech Dev.* 117(1-2): 305-9.

Check E (2007). Stem cells: the hard copy. *Nature.* 446(7135): 485-6.

Chen S, Lewallen M & Xie T (2013). Adhesion in the stem cell niche: biological roles and regulation. *Development.* 140(2): 255-65.

Cheng X, Chen S, Yu X, Zheng P & Wang H (2012). BMP15 gene is activated during human amniotic fluid stem cell differentiation into oocyte-like cells. *DNA Cell Biol.* 31(7): 1198-204.

Childs AJ, Cowan G, Kinnell HL, Anderson RA & Saunders PT (2011). Retinoic Acid signalling and the control of meiotic entry in the human fetal gonad. *PLoS One.* 6(6): e20249.

Childs AJ, Kinnell HL, Collins CS, Hogg K, Bayne RA, Green SJ, Mcneilly AS & Anderson RA (2010). BMP signaling in the human fetal ovary is developmentally regulated and promotes primordial germ cell apoptosis. *Stem Cells.* 28(8): 1368-78.

Childs AJ, Kinnell HL, He J & Anderson RA (2012). LIN28 is selectively expressed by primordial and pre-meiotic germ cells in the human fetal ovary. *Stem Cells Dev.* 21(13): 2343-9.

Chiti MC, Dolmans MM, Orellana R, Soares M, Paulini F, Donnez J & Amorim CA (2016). Influence of follicle stage on artificial ovary outcome using fibrin as a matrix. *Hum Reprod.* 31(2): 427-35.

Cibelli JB, Stice SL, Golueke PJ, Kane JJ, Jerry J, Blackwell C, Ponce De Leon FA & Robl JM (1998). Cloned transgenic calves produced from nonquiescent fetal fibroblasts. *Science.* 280(5367): 1256-8.

Clark AT, Rodriguez RT, Bodnar MS, Abeyta MJ, Cedars MI, Turek PJ, Firpo MT & Reijo Pera RA (2004). Human STELLAR, NANOG, and GDF3 genes are expressed in pluripotent cells and map to chromosome 12p13, a hotspot for teratocarcinoma. *Stem Cells.* 22(2): 169-79.

Cooper GM (2000). *The Cell: A Molecular Approach,* Sunderland (MA), Sinauer Associates.

Coppe JP, Smith AP & Desprez PY (2003). Id proteins in epithelial cells. *Exp Cell Res.* 285(1): 131-45.

Cortvrindt R, Smitz J & Van Steirteghem AC (1996). In-vitro maturation, fertilization and embryo development of immature oocytes from early preantral follicles from prepuberal mice in a simplified culture system. *Hum Reprod.* 11(12): 2656-66.

Couzin-Frankel J (2015). Eggs unlimited. *Science.* 350(6261): 620-4.

Cukierman E, Pankov R, Stevens DR & Yamada KM (2001). Taking cell-matrix adhesions to the third dimension. *Science.* 294(5547): 1708-12.

Damous LL, Nakamuta JS, Saturi De Carvalho AE, Carvalho KC, Soares-Jr JM, Simoes Mde J, Krieger JE & Baracat EC (2015). Scaffold-based delivery of adipose tissue-derived stem cells in rat frozen-thawed ovarian autografts: preliminary studies in a rat model. *J Assist Reprod Genet.* 32(8): 1285-94.

David GFX, Kumar TCA & Baker TG (1974). Uptake of Tritiated-Thymidine by Primordial Germinal Cells in Ovaries of Adult Slender Loris. *Journal of Reproduction and Fertility.* 41(2): 447-51.

Day RN & Davidson MW (2009). The fluorescent protein palette: tools for cellular imaging. *Chem Soc Rev.* 38(10): 2887-921.

De Los Milagros Bassani Molinas M, Beer C, Hesse F, Wirth M & Wagner R (2014). Optimizing the transient transfection process of HEK-293 suspension cells for protein production by nucleotide ratio monitoring. *Cytotechnology.* 66(3): 493-514.

Defelici M, Carlo AD, Pesce M, Iona S, Farrace MG & Piacentini M (1999). Bcl-2 and Bax regulation of apoptosis in germ cells during prenatal oogenesis in the mouse embryo. *Cell Death Differ.* 6(9): 908-15.

Defra. 2016. *Agriculture in the United Kingdom 2015* [Online]. Available: https://www.gov.uk/government/statistics/agriculture-in-the-united-kingdom-2015 [Accessed 26th June 2016].

Dolmans MM, Marinescu C, Saussoy P, Van Langendonckt A, Amorim C & Donnez J (2010). Reimplantation of cryopreserved ovarian tissue from patients with acute lymphoblastic leukemia is potentially unsafe. *Blood.* 116(16): 2908-14.

Donnez J, Dolmans MM, Pellicer A, Diaz-Garcia C, Sanchez Serrano M, Schmidt KT, Ernst E, Luyckx V & Andersen CY (2013). Restoration of ovarian activity and pregnancy after transplantation of cryopreserved ovarian tissue: a review of 60 cases of reimplantation. *Fertil Steril.* 99(6): 1503-13.

Draper BW, Mccallum CM & Moens CB (2007). nanos1 is required to maintain oocyte production in adult zebrafish. *Dev Biol.* 305(2): 589-98.

Dudley BM, Runyan C, Takeuchi Y, Schaible K & Molyneaux K (2007). BMP signaling regulates PGC numbers and motility in organ culture. *Mech Dev.* 124(1): 68-77.

Duke KL (1967). Ovogenetic Activity of Fetal-Type in Ovary of Adult Slow Loris Nycticebus Coucang. *Folia Primatologica.* 7(2): 150-4.

Dunlop CE, Telfer EE & Anderson RA (2013). Ovarian stem cells-Potential roles in infertility treatment and fertility preservation. *Maturitas.* 76(3): 279-83.

Dunlop CE, Telfer EE & Anderson RA (2014). Ovarian germline stem cells. *Stem Cell Research & Therapy.* 5(4): 98.

Eads CA & Laird PW (2002). Combined bisulfite restriction analysis (COBRA). *Methods Mol Biol.* 200: 71-85.

Eggan K, Jurga S, Gosden R, Min IM & Wagers AJ (2006). Ovulated oocytes in adult mice derive from non-circulating germ cells. *Nature.* 441(7097): 1109-14.

El Hajj N, Trapphoff T, Linke M, May A, Hansmann T, Kuhtz J, Reifenberg K, Heinzmann J, Niemann H, Daser A, Eichenlaub-Ritter U, Zechner U & Haaf T (2011). Limiting dilution bisulfite (pyro)sequencing reveals parent-specific methylation patterns in single early mouse embryos and bovine oocytes. *Epigenetics.* 6(10): 1176-88.

Eppig JJ & O'brien MJ (1996). Development in vitro of mouse oocytes from primordial follicles. *Biol Reprod.* 54(1): 197-207.

Eppig JJ & Wigglesworth K (2000). Development of mouse and rat oocytes in chimeric reaggregated ovaries after interspecific exchange of somatic and germ cell components. *Biol Reprod.* 63(4): 1014-23.

Eppig JJ, Wigglesworth K & Pendola FL (2002). The mammalian oocyte orchestrates the rate of ovarian follicular development. *Proc Natl Acad Sci U S A.* 99(5): 2890-4.

Erickson BH (1966). Development and senescence of the postnatal bovine ovary. *J Anim Sci.* 25(3): 800-5.

Evans HM & Swezy O (1932). Ovogenesis and the Normal Follicular Cycle in Adult Mammalia. *Cal West Med.* 36(1): 60.

Ewen-Campen B, Schwager EE & Extavour CG (2010). The molecular machinery of germ line specification. *Mol Reprod Dev.* 77(1): 3-18.

Faddy M & Gosden R (2007). Numbers of ovarian follicles and testing germ line renewal in the postnatal ovary: Facts and fallacies. *Cell Cycle.* 6(15): 1951-2.

Faddy MJ, Telfer E & Gosden RG (1987). The kinetics of pre-antral follicle development in ovaries of CBA/Ca mice during the first 14 weeks of life. *Cell Tissue Kinet.* 20(6): 551-60.

Fakih MH, El Shmoury M, Szeptycki J, Dela Cruz DB, Lux C, Verjee S, Burgess CM, Gabriel MC & Casper RF (2015). The AUGMENTSM Treatment: Physician Reported Outcomes of the Initial Global Patient Experience. *JFIV Reprod Med Genet.* 3(3): http://dx.doi.org/10.4172/2375-4508.1000154.

Farini D, La Sala G, Tedesco M & De Felici M (2007). Chemoattractant action and molecular signaling pathways of Kit ligand on mouse primordial germ cells. *Dev Biol.* 306(2): 572-83.

Fereydouni B, Drummer C, Aeckerle N, Schlatt S & Behr R (2014). The neonatal marmoset monkey ovary is very primitive exhibiting many oogonia. *Reproduction.* 148(2): 237-47.

Fereydouni B, Salinas-Riester G, Heistermann M, Dressel R, Lewerich L, Drummer C & Behr R (2016). Long-Term Oocyte-Like Cell Development in Cultures Derived from Neonatal Marmoset Monkey Ovary. *Stem Cells Int.* 2016: 2480298.

Fujimoto T, Miyayama Y & Fuyuta M (1977). The origin, migration and fine morphology of human primordial germ cells. *Anat Rec.* 188(3): 315-30.

Gimlich RL & Braun J (1985). Improved fluorescent compounds for tracing cell lineage. *Dev Biol.* 109(2): 509-14.

Gittens JE & Kidder GM (2005). Differential contributions of connexin37 and connexin43 to oogenesis revealed in chimeric reaggregated mouse ovaries. *J Cell Sci.* 118(Pt 21): 5071-8.

Godin I, Wylie C & Heasman J (1990). Genital ridges exert long-range effects on mouse primordial germ cell numbers and direction of migration in culture. *Development.* 108(2): 357-63.

Gosden RG (1990). Restitution of fertility in sterilized mice by transferring primordial ovarian follicles. *Hum Reprod.* 5(5): 499-504.

Gosden RG (2004). Germline stem cells in the postnatal ovary: is the ovary more like a testis? *Hum Reprod Update.* 10(3): 193-5.

Goswami D & Conway GS (2007). Premature ovarian failure. *Horm Res.* 68(4): 196-202.

Greenbaum MP, Iwamori T, Buchold GM & Matzuk MM (2011). Germ cell intercellular bridges. *Cold Spring Harb Perspect Biol.* 3(8): a005850.

Greenfeld C & Flaws JA (2004). Renewed debate over postnatal oogenesis in the mammalian ovary. *Bioessays.* 26(8): 829-32.

Greenfeld CR, Pepling ME, Babus JK, Furth PA & Flaws JA (2007). BAX regulates follicular endowment in mice. *Reproduction.* 133(5): 865-76.

Gremel G, Ryan D, Rafferty M, Lanigan F, Hegarty S, Lavelle M, Murphy I, Unwin L, Joyce C, Faller W, Mcdermott EW, Sheahan K, Ponten F & Gallagher WM (2011).

Functional and prognostic relevance of the homeobox protein MSX2 in malignant melanoma. *Br J Cancer.* 105(4): 565-74.

Grondahl ML, Borup R, Vikesa J, Ernst E, Andersen CY & Lykke-Hartmann K (2013). The dormant and the fully competent oocyte: comparing the transcriptome of human oocytes from primordial follicles and in metaphase II. *Mol Hum Reprod.* 19(9): 600-17.

Guan K, Wagner S, Unsold B, Maier LS, Kaiser D, Hemmerlein B, Nayernia K, Engel W & Hasenfuss G (2007). Generation of functional cardiomyocytes from adult mouse spermatogonial stem cells. *Circ Res.* 100(11): 1615-25.

Guo K, Li CH, Wang XY, He DJ & Zheng P (2016). Germ stem cells are active in postnatal mouse ovary under physiological conditions. *Mol Hum Reprod.* 22(5): 316-28.

Hall V, Hinrichs K, Lazzari G, Betts DH & Hyttel P (2013). Early embryonic development, assisted reproductive technologies, and pluripotent stem cell biology in domestic mammals. *Vet J.* 197(2): 128-42.

Handel MA, Eppig JJ & Schimenti JC (2014). Applying "gold standards" to in-vitro-derived germ cells. *Cell.* 157(6): 1257-61.

Hara K, Kanai-Azuma M, Uemura M, Shitara H, Taya C, Yonekawa H, Kawakami H, Tsunekawa N, Kurohmaru M & Kanai Y (2009). Evidence for crucial role of hindgut expansion in directing proper migration of primordial germ cells in mouse early embryogenesis. *Dev Biol.* 330(2): 427-39.

Hartshorne GM, Lyrakou S, Hamoda H, Oloto E & Ghafari F (2009). Oogenesis and cell death in human prenatal ovaries: what are the criteria for oocyte selection? *Mol Hum Reprod.* 15(12): 805-19.

Hawkes K, O'connell JF, Jones NG, Alvarez H & Charnov EL (1998). Grandmothering, menopause, and the evolution of human life histories. *Proc Natl Acad Sci U S A.* 95(3): 1336-9.

Hayashi K, Ogushi S, Kurimoto K, Shimamoto S, Ohta H & Saitou M (2012). Offspring from oocytes derived from in vitro primordial germ cell-like cells in mice. *Science.* 338(6109): 971-5.

He J, Stewart K, Kinnell HL, Anderson RA & Childs AJ (2013). A developmental stage-specific switch from DAZL to BOLL occurs during fetal oogenesis in humans, but not mice. *PLoS One.* 8(9): e73996.

Heinzmann J, Hansmann T, Herrmann D, Wrenzycki C, Zechner U, Haaf T & Niemann H (2011). Epigenetic profile of developmentally important genes in bovine oocytes. *Mol Reprod Dev.* 78(3): 188-201.

Henderson JK, Draper JS, Baillie HS, Fishel S, Thomson JA, Moore H & Andrews PW (2002). Preimplantation human embryos and embryonic stem cells show comparable expression of stage-specific embryonic antigens. *Stem Cells.* 20(4): 329-37.

Henderson SA & Edwards RG (1968). Chiasma frequency and maternal age in mammals. *Nature.* 218(5136): 22-8.

Hermann BP, Phillips BT & Orwig KE (2011). The elusive spermatogonial stem cell marker? *Biol Reprod.* 85(2): 221-3.

Hernandez SF, Vahidi NA, Park S, Weitzel RP, Tisdale J, Rueda BR & Wolff EF (2015). Characterization of extracellular DDX4- or Ddx4-positive ovarian cells. *Nat Med.* 21(10): 1114-6.

Herrera B & Inman GJ (2009). A rapid and sensitive bioassay for the simultaneous measurement of multiple bone morphogenetic proteins. Identification and quantification of BMP4, BMP6 and BMP9 in bovine and human serum. *BMC Cell Biol.* 10: 20.

Hickford DE, Frankenberg S, Pask AJ, Shaw G & Renfree MB (2011). DDX4 (VASA) is conserved in germ cell development in marsupials and monotremes. *Biol Reprod.* 85(4): 733-43.

Hines WC, Su Y, Kuhn I, Polyak K & Bissell MJ (2014). Sorting out the FACS: a devil in the details. *Cell Rep.* 6(5): 779-81.

Hirai H, Karian P & Kikyo N (2011). Regulation of embryonic stem cell self-renewal and pluripotency by leukaemia inhibitory factor. *Biochem J.* 438(1): 11-23.

Hirao Y, Nagai T, Kubo M, Miyano T, Miyake M & Kato S (1994). In vitro growth and maturation of pig oocytes. *J Reprod Fertil.* 100(2): 333-9.

Hirshfield AN (1984). Continuous [3H] thymidine infusion: a method for the study of follicular dynamics. *Biol Reprod.* 30(2): 485-91.

Hirshfield AN (1994). Relationship between the supply of primordial follicles and the onset of follicular growth in rats. *Biol Reprod.* 50(2): 421-8.

Hogan BL (1996). Bone morphogenetic proteins in development. *Curr Opin Genet Dev.* 6(4): 432-8.

Hollnagel A, Oehlmann V, Heymer J, Ruther U & Nordheim A (1999). Id genes are direct targets of bone morphogenetic protein induction in embryonic stem cells. *J Biol Chem.* 274(28): 19838-45.

Horie K, Fujita J, Takakura K, Kanzaki H, Suginami H, Iwai M, Nakayama H & Mori T (1993). The expression of c-kit protein in human adult and fetal tissues. *Hum Reprod.* 8(11): 1955-62.

Hou J, Niu M, Liu L, Zhu Z, Wang X, Sun M, Yuan Q, Yang S, Zeng W, Liu Y, Li Z & He Z (2015). Establishment and Characterization of Human Germline Stem Cell Line with Unlimited Proliferation Potentials and no Tumor Formation. *Sci Rep.* 5: 16922.

Houdebine LM (2007). Transgenic animal models in biomedical research. *Methods Mol Biol.* 360: 163-202.

Hoyer PE, Byskov AG & Mollgard K (2005). Stem cell factor and c-Kit in human primordial germ cells and fetal ovaries. *Mol Cell Endocrinol.* 234(1-2): 1-10.

Hu Y, Bai Y, Chu Z, Wang J, Wang L, Yu M, Lian Z & Hua J (2012). GSK3 inhibitor-BIO regulates proliferation of female germline stem cells from the postnatal mouse ovary. *Cell Prolif.* 45(4): 287-98.

Hummitzsch K, Anderson RA, Wilhelm D, Wu J, Telfer EE, Russell DL, Robertson SA & Rodgers RJ (2015). Stem cells, progenitor cells, and lineage decisions in the ovary. *Endocr Rev.* 36(1): 65-91.

Hutt KJ (2015). The role of BH3-only proteins in apoptosis within the ovary. *Reproduction.* 149(2): R81-9.

Imudia AN, Wang N, Tanaka Y, White YA, Woods DC & Tilly JL (2013). Comparative gene expression profiling of adult mouse ovary-derived oogonial stem cells supports a distinct cellular identity. *Fertil Steril.* 100(5): 1451-8.

Ioannou JM (1967). Oogenesis in adult prosimians. *J Embryol Exp Morphol.* 17(1): 139-45.

Irie N, Tang WW & Azim Surani M (2014). Germ cell specification and pluripotency in mammals: a perspective from early embryogenesis. *Reprod Med Biol.* 13(4): 203-215.

Irie N, Weinberger L, Tang WW, Kobayashi T, Viukov S, Manor YS, Dietmann S, Hanna JH & Surani MA (2015). SOX17 is a critical specifier of human primordial germ cell fate. *Cell.* 160(1-2): 253-68.

Jenkins HP, Senz EH & Et Al. (1946). Present status of gelatin sponge for the control of hemorrhage; with experimental data on its use for wounds of the great vessels and the heart. *J Am Med Assoc.* 132(11): 614-9.

John GB, Shirley LJ, Gallardo TD & Castrillon DH (2007). Specificity of the requirement for Foxo3 in primordial follicle activation. *Reproduction.* 133(5): 855-63.

Johnson J, Bagley J, Skaznik-Wikiel M, Lee HJ, Adams GB, Niikura Y, Tschudy KS, Tilly JC, Cortes ML, Forkert R, Spitzer T, Iacomini J, Scadden DT & Tilly JL (2005a). Oocyte generation in adult mammalian ovaries by putative germ cells in bone marrow and peripheral blood. *Cell.* 122(2): 303-15.

Johnson J, Canning J, Kaneko T, Pru JK & Tilly JL (2004). Germline stem cells and follicular renewal in the postnatal mammalian ovary. *Nature.* 428(6979): 145-50.

Johnson J, Skaznik-Wikiel M, Lee HJ, Niikura Y, Tilly JC & Tilly JL (2005b). Setting the record straight on data supporting postnatal oogenesis in female mammals. *Cell Cycle.* 4(11): 1471-7.

Jorgensen A, Nielsen JE, Perlman S, Lundvall L, Mitchell RT, Juul A & Rajpert-De Meyts E (2015). Ex vivo culture of human fetal gonads: manipulation of meiosis signalling by retinoic acid treatment disrupts testis development. *Hum Reprod.* 30(10): 2351-63.

Jung D & Kee K (2015). Insights into female germ cell biology: from in vivo development to in vitro derivations. *Asian J Androl.* 17(3): 415-20.

Kagawa N, Silber S & Kuwayama M (2009). Successful vitrification of bovine and human ovarian tissue. *Reprod Biomed Online.* 18: 568-77.

Kakiuchi K, Tsuda A, Goto Y, Shimada T, Taniguchi K, Takagishi K & Kubota H (2014). Cell-surface DEAD-box polypeptide 4-immunoreactive cells and gonocytes are two distinct populations in postnatal porcine testes. *Biol Reprod.* 90(4): 82.

Katagiri T & Watabe T (2016). Bone Morphogenetic Proteins. *Cold Spring Harb Perspect Biol.* 8(6).

Kaushik R, Singh KP, Bahuguna V, Rameshbabu K, Singh MK, Manik RS, Palta P, Singla SK & Chauhan MS (2015). Molecular characterization and expression of buffalo (Bubalus bubalis) DEAD-box family VASA gene and mRNA transcript variants isolated from testis tissue. *Gene.* 572(1): 17-26.

Kee K, Angeles VT, Flores M, Nguyen HN & Reijo Pera RA (2009). Human DAZL, DAZ and BOULE genes modulate primordial germ-cell and haploid gamete formation. *Nature.* 462(7270): 222-5.

Kee K, Gonsalves JM, Clark AT & Pera RA (2006). Bone morphogenetic proteins induce germ cell differentiation from human embryonic stem cells. *Stem Cells Dev.* 15(6): 831-7.

Kehler J, Tolkunova E, Koschorz B, Pesce M, Gentile L, Boiani M, Lomeli H, Nagy A, Mclaughlin KJ, Scholer HR & Tomilin A (2004). Oct4 is required for primordial germ cell survival. *EMBO Rep.* 5(11): 1078-83.

Kent L (2009). Freezing and Thawing Human Embryonic Stem Cells. *Journal of Visualized Experiments.* 34. http://www.jove.com/details.php?id=1555, doi: 10.3791/1555

Kerr JB, Duckett R, Myers M, Britt KL, Mladenovska T & Findlay JK (2006). Quantification of healthy follicles in the neonatal and adult mouse ovary: evidence for maintenance of primordial follicle supply. *Reproduction.* 132(1): 95-109.

Kimble J (2011). Molecular regulation of the mitosis/meiosis decision in multicellular organisms. *Cold Spring Harb Perspect Biol.* 3(8): a002683.

Kimble JE & White JG (1981). On the control of germ cell development in Caenorhabditis elegans. *Dev Biol.* 81(2): 208-19.

Kniazeva E, Hardy AN, Boukaidi SA, Woodruff TK, Jeruss JS & Shea LD (2015). Primordial Follicle Transplantation within Designer Biomaterial Grafts Produce Live Births in a Mouse Infertility Model. *Sci Rep.* 5: 17709.

Kodaira K, Imada M, Goto M, Tomoyasu A, Fukuda T, Kamijo R, Suda T, Higashio K & Katagiri T (2006). Purification and identification of a BMP-like factor from bovine serum. *Biochem Biophys Res Commun.* 345(3): 1224-31.

Kolasa A, Misiakiewicz K, Marchlewicz M & Wizniewska B (2011). The generation of spermatogonial stem cells and spermatogonia in mammals. *Reprod Biol.* 12(1): 5 - 23.

Kondapalli LA, Dillon KE, Sammel MD, Ray A, Prewitt M, Ginsberg JP & Gracia CR (2014). Quality of life in female cancer survivors: is it related to ovarian reserve? *Qual Life Res.* 23(2): 585-92.

Koubova J, Menke DB, Zhou Q, Capel B, Griswold MD & Page DC (2006). Retinoic acid regulates sex-specific timing of meiotic initiation in mice. *Proc Natl Acad Sci U S A.* 103(8): 2474-9.

Krotz SP, Robins JC, Ferruccio TM, Moore R, Steinhoff MM, Morgan JR & Carson S (2010). In vitro maturation of oocytes via the pre-fabricated self-assembled artificial human ovary. *J Assist Reprod Genet.* 27(12): 743-50.

Kuo LJ & Yang LX (2008). Gamma-H2AX - a novel biomarker for DNA double-strand breaks. *In Vivo.* 22(3): 305-9.

Lai D, Wang F, Yao X, Zhang Q, Wu X & Xiang C (2015). Human endometrial mesenchymal stem cells restore ovarian function through improving the renewal of germline stem cells in a mouse model of premature ovarian failure. *J Transl Med.* 13: 155.

Lange UC, Adams DJ, Lee C, Barton S, Schneider R, Bradley A & Surani MA (2008). Normal germ line establishment in mice carrying a deletion of the Ifitm/Fragilis gene family cluster. *Mol Cell Biol.* 28(15): 4688-96.

Lange UC, Saitou M, Western PS, Barton SC & Surani MA (2003). The fragilis interferon-inducible gene family of transmembrane proteins is associated with germ cell specification in mice. *BMC Dev Biol.* 3: 1.

Lanigan F, Gremel G, Hughes R, Brennan DJ, Martin F, Jirstrom K & Gallagher WM (2010). Homeobox transcription factor muscle segment homeobox 2 (Msx2) correlates with good prognosis in breast cancer patients and induces apoptosis in vitro. *Breast Cancer Res.* 12(4): R59.

Laronda MM, Duncan FE, Hornick JE, Xu M, Pahnke JE, Whelan KA, Shea LD & Woodruff TK (2014). Alginate encapsulation supports the growth and differentiation of human primordial follicles within ovarian cortical tissue. *J Assist Reprod Genet.* 31(8): 1013-28.

Laronda MM, Jakus AE, Whelan KA, Wertheim JA, Shah RN & Woodruff TK (2015). Initiation of puberty in mice following decellularized ovary transplant. *Biomaterials.* 50: 20-9.

Laronda MM, Rutz AL, Xiao S, Whelan KA, Shah RN & Woodruff TK (2016). A 3D Printed Ovarian Bioprosthesis Restores Estrous Cyclicity and Supports Natural Ovulation, Live Birth and Lactation LB-OR01-1 [Abstract]. *Endocr Rev.* 37(2 Suppl).

Lawson KA, Dunn NR, Roelen BA, Zeinstra LM, Davis AM, Wright CV, Korving JP & Hogan BL (1999). Bmp4 is required for the generation of primordial germ cells in the mouse embryo. *Genes Dev.* 13(4): 424-36.

Le Bouffant R, Guerquin MJ, Duquenne C, Frydman N, Coffigny H, Rouiller-Fabre V, Frydman R, Habert R & Livera G (2010). Meiosis initiation in the human ovary requires intrinsic retinoic acid synthesis. *Hum Reprod.* 25(10): 2579-90.

Le Bouffant R, Souquet B, Duval N, Duquenne C, Herve R, Frydman N, Robert B, Habert R & Livera G (2011). Msx1 and Msx2 promote meiosis initiation. *Development.* 138(24): 5393-402.

Lee HJ, Selesniemi K, Niikura Y, Niikura T, Klein R, Dombkowski DM & Tilly JL (2007). Bone marrow transplantation generates immature oocytes and rescues long-term fertility in a preclinical mouse model of chemotherapy-induced premature ovarian failure. *J Clin Oncol.* 25(22): 3198-204.

Lee JY, Choi MH, Shin EY & Kang YK (2011). Autologous mesenchymal stem cells loaded in Gelfoam((R)) for structural bone allograft healing in rabbits. *Cell Tissue Bank.* 12(4): 299-309.

Lefievre L, Conner SJ, Salpekar A, Olufowobi O, Ashton P, Pavlovic B, Lenton W, Afnan M, Brewis IA, Monk M, Hughes DC & Barratt CL (2004). Four zona pellucida glycoproteins are expressed in the human. *Hum Reprod.* 19(7): 1580-6.

Lei L & Spradling AC (2013). Female mice lack adult germ-line stem cells but sustain oogenesis using stable primordial follicles. *Proceedings of the National Academy of Sciences of the United States of America.* 110(21): 8585-8590.

Li R & Albertini DF (2013). The road to maturation: somatic cell interaction and self-organization of the mammalian oocyte. *Nat Rev Mol Cell Biol.* 14(3): 141-52.

Lin IY, Chiu FL, Yeang CH, Chen HF, Chuang CY, Yang SY, Hou PS, Sintupisut N, Ho HN, Kuo HC & Lin KI (2014). Suppression of the SOX2 neural effector gene by PRDM1 promotes human germ cell fate in embryonic stem cells. *Stem Cell Reports.* 2(2): 189-204.

Lin Y, Gill ME, Koubova J & Page DC (2008). Germ cell-intrinsic and -extrinsic factors govern meiotic initiation in mouse embryos. *Science.* 322(5908): 1685-7.

Linher K, Dyce P & Li J (2009). Primordial germ cell-like cells differentiated in vitro from skin-derived stem cells. *PLoS One.* 4(12): e8263.

Liu Y, Wu C, Lyu Q, Yang D, Albertini DF, Keefe DL & Liu L (2007). Germline stem cells and neo-oogenesis in the adult human ovary. *Dev Biol.* 306(1): 112-20.

Livak KJ & Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods.* 25(4): 402-8.

Loi P, Ptak G, Barboni B, Fulka J, Jr., Cappai P & Clinton M (2001). Genetic rescue of an endangered mammal by cross-species nuclear transfer using post-mortem somatic cells. *Nat Biotechnol.* 19(10): 962-4.

Lu Z, Wu M, Zhang J, Xiong J, Cheng J, Shen W, Luo A, Fang L & Wang S (2016). Improvement in Isolation and Identification of Mouse Oogonial Stem Cells. *Stem Cells Int.* 2016: 2749461.

Luo H, Zhou Y, Li Y & Li Q (2013). Splice variants and promoter methylation status of the Bovine Vasa Homology (Bvh) gene may be involved in bull spermatogenesis. *BMC Genet.* 14: 58.

Luyckx V, Dolmans MM, Vanacker J, Legat C, Fortuno Moya C, Donnez J & Amorim CA (2014). A new step toward the artificial ovary: survival and proliferation of isolated murine follicles after autologous transplantation in a fibrin scaffold. *Fertil Steril.* 101(4): 1149-56.

Luyckx V, Dolmans MM, Vanacker J, Scalercio SR, Donnez J & Amorim CA (2013). First step in developing a 3D biodegradable fibrin scaffold for an artificial ovary. *J Ovarian Res.* 6(1): 83.

Ma Z, Liu R, Wang X, Huang M, Gao Q, Lu Y & Liu C (2013). Spontaneous germline potential of human hepatic cell line in vitro. *Mol Hum Reprod.* 19(4): 216-26.

Magnani C, Pastore G, Coebergh JW, Viscomi S, Spix C & Steliarova-Foucher E (2006). Trends in survival after childhood cancer in Europe, 1978-1997: report from the Automated Childhood Cancer Information System project (ACCIS). *Eur J Cancer.* 42(13): 1981-2005.

Manova K & Bachvarova RF (1991). Expression of c-kit encoded at the W locus of mice in developing embryonic germ cells and presumptive melanoblasts. *Dev Biol.* 146(2): 312-24.

Marazzi G, Wang Y & Sassoon D (1997). Msx2 is a transcriptional regulator in the BMP4-mediated programmed cell death pathway. *Dev Biol.* 186(2): 127-38.

Maruotti J, Munoz M, Degrelle SA, Gomez E, Louet C, Diez C, De Longchamp PH, Brochard V, Hue I, Caamano JN & Jouneau A (2012). Efficient derivation of bovine embryonic stem cells needs more than active core pluripotency factors. *Mol Reprod Dev.* 79(7): 461-77.

McLaren A & Southee D (1997). Entry of mouse embryonic germ cells into meiosis. *Dev Biol.* 187(1): 107-13.

McLaughlin M, Kelsey TW, Wallace WH, Anderson RA & Telfer EE (2015). An externally validated age-related model of mean follicle density in the cortex of the human ovary. *J Assist Reprod Genet.* 32(7): 1089-95.

McLaughlin M, Kelsey TW, Wallace WH, Anderson RA & Telfer EE (2016). ABVD chemotherapy for lymphoma affects number and morphology of primordial follicles in the adolescent and adult ovary O-287 [Abstract]. *Hum Reprod.* 31(Suppl. 1).

McLaughlin M & Telfer EE (2010). Oocyte development in bovine primordial follicles is promoted by activin and FSH within a two-step serum-free culture system. *Reproduction.* 139(6): 971-8.

Medrano JV, Ramathal C, Nguyen HN, Simon C & Reijo Pera RA (2012). Divergent RNA-binding proteins, DAZL and VASA, induce meiotic progression in human germ cells derived in vitro. *Stem Cells.* 30(3): 441-51.

Mitsui K, Tokuzawa Y, Itoh H, Segawa K, Murakami M, Takahashi K, Maruyama M, Maeda M & Yamanaka S (2003). The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell.* 113(5): 631-42.

Molyneaux KA, Zinszner H, Kunwar PS, Schaible K, Stebler J, Sunshine MJ, O'brien W, Raz E, Littman D, Wylie C & Lehmann R (2003). The chemokine SDF1/CXCL12 and its receptor CXCR4 regulate mouse germ cell migration and survival. *Development.* 130(18): 4279-86.

Moss EG & Tang L (2003). Conservation of the heterochronic regulator Lin-28, its developmental expression and microRNA complementary sites. *Dev Biol.* 258(2): 432-42.

Motta PM, Nottola SA & Makabe S (1997). Natural history of the female germ cell from its origin to full maturation through prenatal ovarian development. *Eur J Obstet Gynecol Reprod Biol.* 75(1): 5-10.

Nakamura S, Kobayashi K, Nishimura T, Higashijima S & Tanaka M (2010). Identification of germline stem cells in the ovary of the teleost medaka. *Science.* 328(5985): 1561-3.

Nakamura T, Arai Y, Umehara H, Masuhara M, Kimura T, Taniguchi H, Sekimoto T, Ikawa M, Yoneda Y, Okabe M, Tanaka S, Shiota K & Nakano T (2007). PGC7/Stella protects against DNA demethylation in early embryogenesis. *Nat Cell Biol.* 9(1): 64-71.

Naldini L, Blomer U, Gallay P, Ory D, Mulligan R, Gage FH, Verma IM & Trono D (1996). In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science.* 272(5259): 263-7.

Nation A & Selwood L (2009). The production of mature oocytes from adult ovaries following primary follicle culture in a marsupial. *Reproduction.* 138(2): 247-55.

Nelsen SM & Christian JL (2009). Site-specific cleavage of BMP4 by furin, PC6, and PC7. *J Biol Chem.* 284(40): 27157-66.

Nelson HD (2008). Menopause. *Lancet.* 371(9614): 760-70.

Nelson SM, Telfer EE & Anderson RA (2013). The ageing ovary and uterus: new biological insights. *Hum Reprod Update.* 19(1): 67-83.

Nichols J, Zevnik B, Anastassiadis K, Niwa H, Klewe-Nebenius D, Chambers I, Scholer H & Smith A (1998). Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell.* 95(3): 379-91.

Nigten J, Breems-De Ridder MC, Erpelinck-Verschueren CA, Nikoloski G, Van Der Reijden BA, Van Wageningen S, Van Hennik PB, De Witte T, Lowenberg B & Jansen

JH (2005). ID1 and ID2 are retinoic acid responsive genes and induce a G0/G1 accumulation in acute promyelocytic leukemia cells. *Leukemia.* 19(5): 799-805.

Niikura Y, Niikura T & Tilly JL (2009). Aged mouse ovaries possess rare premeiotic germ cells that can generate oocytes following transplantation into a young host environment. *Aging (Albany NY).* 1(12): 971-8.

Nishiyama K, Takaji K, Uchijima Y, Kurihara Y, Asano T, Yoshimura M, Ogawa H & Kurihara H (2007). Protein kinase A-regulated nucleocytoplasmic shuttling of Id1 during angiogenesis. *J Biol Chem.* 282(23): 17200-9.

Nong W, Xie TS, Li LY, Lu AG, Mo J, Gou YF, Lan G, Jiang H, Len J, Li MM, Jiang QY & Huang B (2015). Qualitative Analyses of Protein Phosphorylation in Bovine Pluripotent Stem Cells Generated from Embryonic Fibroblasts. *Reprod Domest Anim.* 50(6): 989-98.

Notarianni E (2011). Reinterpretation of evidence advanced for neo-oogenesis in mammals, in terms of a finite oocyte reserve. *J Ovarian Res.* 4(1): 1.

O'Brien MJ, Pendola JK & Eppig JJ (2003). A revised protocol for in vitro development of mouse oocytes from primordial follicles dramatically improves their developmental competence. *Biol Reprod.* 68(5): 1682-6.

O W-S & Baker TG (1978). Germinal and somatic cell interrelationships in gonadal sex differentiation. *Ann Biol anim Bioch Biophys.* 18(2B): 351-357.

Oatley J & Hunt PA (2012). Of mice and (wo)men: purified oogonial stem cells from mouse and human ovaries. *Biol Reprod.* 86(6): 196.

Ohinata Y, Ohta H, Shigeta M, Yamanaka K, Wakayama T & Saitou M (2009). A signaling principle for the specification of the germ cell lineage in mice. *Cell.* 137(3): 571-84.

Okamoto K, Okazawa H, Okuda A, Sakai M, Muramatsu M & Hamada H (1990). A Novel Octamer Binding Transcription Factor Is Differentially Expressed in Mouse Embryonic-Cells. *Cell.* 60(3): 461-472.

Okamura D, Tokitake Y, Niwa H & Matsui Y (2008). Requirement of Oct3/4 function for germ cell specification. *Dev Biol.* 317(2): 576-84.

Oktay K (2006). Spontaneous conceptions and live birth after heterotopic ovarian transplantation: is there a germline stem cell connection? *Hum Reprod.* 21(6): 1345-8.

Oktay K, Turkcuoglu I & Rodriguez-Wallberg KA (2011). Four spontaneous pregnancies and three live births following subcutaneous transplantation of frozen banked ovarian tissue: what is the explanation? *Fertil Steril.* 95(2): 804 e7-10.

Pacchiarotti J, Maki C, Ramos T, Marh J, Howerton K, Wong J, Pham J, Anorve S, Chow YC & Izadyar F (2010). Differentiation potential of germ line stem cells derived from the postnatal mouse ovary. *Differentiation.* 79(3): 159-70.

Pan L, Chen SY, Weng CJ, Call G, Zhu DX, Tang H, Zhang N & Xie T (2007). Stem cell aging is controlled both intrinsically and extrinsically in the Drosophila ovary. *Cell Stem Cell.* 1(4): 458-469.

Panay N & Kalu E (2009). Management of premature ovarian failure. *Best Pract Res Clin Obstet Gynaecol.* 23(1): 129-40.

Park ES & Tilly JL (2015). Use of DEAD-box polypeptide-4 (Ddx4) gene promoter-driven fluorescent reporter mice to identify mitotically active germ cells in post-natal mouse ovaries. *Mol Hum Reprod.* 21(1): 58-65.

Park ES, Woods DC & Tilly JL (2013). Bone morphogenetic protein 4 promotes mammalian oogonial stem cell differentiation via Smad1/5/8 signaling. *Fertil Steril.* 100(5): 1468-1475 e2.

Parte S, Bhartiya D, Telang J, Daithankar V, Salvi V, Zaveri K & Hinduja I (2011). Detection, characterization, and spontaneous differentiation in vitro of very small embryonic-like putative stem cells in adult mammalian ovary. *Stem Cells Dev.* 20(8): 1451-64.

Paulini F, Vilela JM, Chiti MC, Donnez J, Jadoul P, Dolmans MM & Amorim CA (2016). Survival and growth of human preantral follicles after cryopreservation of ovarian tissue, follicle isolation and short-term xenografting. *Reprod Biomed Online.* 33(3): 425-32.

Pennetier S, Uzbekova S, Perreau C, Papillier P, Mermillod P & Dalbies-Tran R (2004). Spatio-temporal expression of the germ cell marker genes MATER, ZAR1, GDF9, BMP15,andVASA in adult bovine tissues, oocytes, and preimplantation embryos. *Biol Reprod.* 71(4): 1359-66.

Pepling ME & Spradling AC (1998). Female mouse germ cells form synchronously dividing cysts. *Development.* 125(17): 3323-8.

Pepling ME & Spradling AC (2001). Mouse ovarian germ cell cysts undergo programmed breakdown to form primordial follicles. *Dev Biol.* 234(2): 339-51.

Perrett RM, Turnpenny L, Eckert JJ, O'shea M, Sonne SB, Cameron IT, Wilson DI, Rajpert-De Meyts E & Hanley NA (2008). The early human germ cell lineage does not express SOX2 during in vivo development or upon in vitro culture. *Biol Reprod.* 78(5): 852-8.

Pesce M, Gross MK & Scholer HR (1998). In line with our ancestors: Oct-4 and the mammalian germ. *Bioessays.* 20(9): 722-32.

Ponticiello MS, Schinagl RM, Kadiyala S & Barry FP (2000). Gelatin-based resorbable sponge as a carrier matrix for human mesenchymal stem cells in cartilage regeneration therapy. *J Biomed Mater Res.* 52(2): 246-55.

Powell K (2007). Going against the grain. *PLoS Biol.* 5(12): e338.

Qing T, Liu H, Wei W, Ye X, Shen W, Zhang D, Song Z, Yang W, Ding M & Deng H (2008). Mature oocytes derived from purified mouse fetal germ cells. *Hum Reprod.* 23(1): 54-61.

Qing T, Shi Y, Qin H, Ye X, Wei W, Liu H, Ding M & Deng H (2007). Induction of oocyte-like cells from mouse embryonic stem cells by co-culture with ovarian granulosa cells. *Differentiation.* 75(10): 902-11.

Rasweiler JJT (1972). Reproduction in the long-tongued bat, Glossophaga soricina. I. Preimplantation development and histology of the oviduct. *J Reprod Fertil.* 31(2): 249-62.

Ratts VS, Flaws JA, Kolp R, Sorenson CM & Tilly JL (1995). Ablation of bcl-2 gene expression decreases the numbers of oocytes and primordial follicles established in the post-natal female mouse gonad. *Endocrinology.* 136(8): 3665-8.

Regaud C (1901a). Etudes sur la structure des tubes seminiferes et sur la spermatogenese chez les mammiferes. Part 1. *Archives d'Anatomie microscopiques et de Morphologie experimentale.* 4: 101-156.

Regaud C (1901b). Etudes sur la structure des tubes seminiferes et sur la spermatogenese chez les mammiferes. Part 2. *Archives d'Anatomie microscopiques et de Morphologie experimentale.* 4: 231-280.

Reizel Y, Itzkovitz S, Adar R, Elbaz J, Jinich A, Chapal-Ilani N, Maruvka YE, Nevo N, Marx Z, Horovitz I, Wasserstrom A, Mayo A, Shur I, Benayahu D, Skorecki K, Segal E, Dekel N & Shapiro E (2012). Cell lineage analysis of the mammalian female germline. *PLoS Genet.* 8(2): e1002477.

Reynolds N, Collier B, Bingham V, Gray NK & Cooke HJ (2007). Translation of the synaptonemal complex component Sycp3 is enhanced in vivo by the germ cell specific regulator Dazl. *RNA.* 13(7): 974-81.

Rhinn M & Dolle P (2012). Retinoic acid signalling during development. *Development.* 139(5): 843-58.

Richards M, Tan SP, Tan JH, Chan WK & Bongso A (2004). The transcriptome profile of human embryonic stem cells as defined by SAGE. *Stem Cells.* 22(1): 51-64.

Robinson A (1918). The formation, rupture and closure of ovarian follicles in ferrets and ferret-polecat, hybrids, and some associated phenomena. *Transactions of the Royal Society of Edinburgh.* 52(Part 2, no. XXIII): 303 - 362.

Robinson LL, Gaskell TL, Saunders PT & Anderson RA (2001). Germ cell specific expression of c-kit in the human fetal gonad. *Mol Hum Reprod.* 7(9): 845-52.

Rodgers RJ, Irving-Rodgers HF & Russell DL (2003). Extracellular matrix of the developing ovarian follicle. *Reproduction.* 126(4): 415-24.

Rosendahl M, Andersen MT, Ralfkiaer E, Kjeldsen L, Andersen MK & Andersen CY (2010). Evidence of residual disease in cryopreserved ovarian cortex from female patients with leukemia. *Fertil Steril.* 94(6): 2186-90.

Rosner MH, Vigano MA, Ozato K, Timmons PM, Poirier F, Rigby PW & Staudt LM (1990). A POU-domain transcription factor in early stem cells and germ cells of the mammalian embryo. *Nature.* 345(6277): 686-92.

Rowsey R, Gruhn J, Broman KW, Hunt PA & Hassold T (2014). Examining variation in recombination levels in the human female: a test of the production-line hypothesis. *Am J Hum Genet.* 95(1): 108-12.

Rudkin GT & Griech HA (1962). On the persistence of oocyte nuclei from fetus to maturity in the laboratory mouse. *J Cell Biol.* 12: 169-75.

Ruggiu M, Speed R, Taggart M, Mckay SJ, Kilanowski F, Saunders P, Dorin J & Cooke HJ (1997). The mouse Dazla gene encodes a cytoplasmic protein essential for gametogenesis. *Nature.* 389(6646): 73-7.

Saiti D & Lacham-Kaplan O (2007). Mouse Germ Cell Development in-vivo and in-vitro. *Biomark Insights.* 2: 241-52.

Saitou M, Barton SC & Surani MA (2002). A molecular programme for the specification of germ cell fate in mice. *Nature.* 418(6895): 293-300.

Sakai T, Li RK, Weisel RD, Mickle DA, Kim ET, Jia ZQ & Yau TM (2001). The fate of a tissue-engineered cardiac graft in the right ventricular outflow tract of the rat. *J Thorac Cardiovasc Surg.* 121(5): 932-42.

Sanchez-Serrano M, Crespo J, Mirabet V, Cobo AC, Escriba MJ, Simon C & Pellicer A (2010). Twins born after transplantation of ovarian cortical tissue and oocyte vitrification. *Fertil Steril.* 93(1): 268 e11-3.

Sarraj MA & Drummond AE (2012). Mammalian foetal ovarian development: consequences for health and disease. *Reproduction.* 143(2): 151-63.

Saunders PT, Turner JM, Ruggiu M, Taggart M, Burgoyne PS, Elliott D & Cooke HJ (2003). Absence of mDazl produces a final block on germ cell development at meiosis. *Reproduction.* 126(5): 589-97.

Sharma GT, Dubey PK & Meur SK (2009). Survival and developmental competence of buffalo preantral follicles using three-dimensional collagen gel culture system. *Anim Reprod Sci.* 114(1-3): 115-24.

Shim SW, Han DW, Yang JH, Lee BY, Kim SB, Shim H & Lee HT (2008). Derivation of embryonic germ cells from post migratory primordial germ cells, and methylation analysis of their imprinted genes by bisulfite genomic sequencing. *Mol Cells.* 25(3): 358-67.

Signer RA & Morrison SJ (2013). Mechanisms that regulate stem cell aging and life span. *Cell Stem Cell.* 12(2): 152-65.

Smith A (1947). Absorbable Gelatin sponge - new and nonofficial remedies. *JAMA.* 135: 921.

Smith C, Berg D, Beaumont S, Standley NT, Wells DN & Pfeffer PL (2007). Simultaneous gene quantitation of multiple genes in individual bovine nuclear transfer blastocysts. *Reproduction.* 133(1): 231-42.

Soares M, Sahrari K, Chiti MC, Amorim CA, Ambroise J, Donnez J & Dolmans MM (2015). The best source of isolated stromal cells for the artificial ovary: medulla or cortex, cryopreserved or fresh? *Hum Reprod.* 30(7): 1589-98.

Sowers MR & La Pietra MT (1995). Menopause: its epidemiology and potential association with chronic diseases. *Epidemiol Rev.* 17(2): 287-302.

Spradling A, Fuller MT, Braun RE & Yoshida S (2011). Germline stem cells. *Cold Spring Harb Perspect Biol.* 3(11): a002642.

Stone R (1999). Paleontology. Siberian mammoth find raises hopes, questions. *Science.* 286(5441): 876-7.

Stoop H, Honecker F, Cools M, De Krijger R, Bokemeyer C & Looijenga LH (2005). Differentiation and development of human female germ cells during prenatal gonadogenesis: an immunohistochemical study. *Hum Reprod.* 20(6): 1466-76.

Sugawa F, Arauzo-Bravo MJ, Yoon J, Kim KP, Aramaki S, Wu G, Stehling M, Psathaki OE, Hubner K & Scholer HR (2015). Human primordial germ cell commitment in vitro associates with a unique PRDM14 expression profile. *EMBO J.* 34(8): 1009-24.

Sun M, Wang S, Li Y, Yu L, Gu F, Wang C & Yao Y (2013). Adipose-derived stem cells improved mouse ovary function after chemotherapy-induced ovary failure. *Stem Cell Res Ther.* 4(4): 80.

Suzumori N, Yan C, Matzuk MM & Rajkovic A (2002). Nobox is a homeobox-encoding gene preferentially expressed in primordial and growing oocytes. *Mech Dev.* 111(1-2): 137-41.

Tagler DJ, Shea LD & Woodruff TK 2011. Contribution of ovarian stromal cells to follicle culture. *In:* DONNEZ, J. & KIM, S. S. (eds.) *In: Principles and Practice of Fertility Preservation.* 1st ed. Cambridge: Cambridge University Press.

Tanaka SS & Matsui Y (2002). Developmentally regulated expression of mil-1 and mil-2, mouse interferon-induced transmembrane protein like genes, during formation and differentiation of primordial germ cells. *Mech Dev.* 119 Suppl 1: S261-7.

Tedesco M, Desimio MG, Klinger FG, De Felici M & Farini D (2013). Minimal concentrations of retinoic acid induce stimulation by retinoic acid 8 and promote entry into meiosis in isolated pregonadal and gonadal mouse primordial germ cells. *Biol Reprod.* 88(6): 145.

Telfer EE & Albertini DF (2012). The quest for human ovarian stem cells. *Nat Med.* 18(3): 353-4.

Telfer EE, Gosden RG, Byskov AG, Spears N, Albertini D, Andersen CY, Anderson R, Braw-Tal R, Clarke H, Gougeon A, Mclaughlin E, Mclaren A, Mcnatty K, Schatten G, Silber S & Tsafriri A (2005). On regenerating the ovary and generating controversy. *Cell.* 122(6): 821-2.

Telfer EE & Mclaughlin M 2011. In vitro growth systems for human oocytes: from primordial to maturation. *In:* DONNEZ, J. & KIM, S. S. (eds.) *Principles and Practice of Fertility Preservation.* 1st ed. Cambridge: Cambridge University Press.

Telfer EE, Mclaughlin M, Ding C & Thong KJ (2008). A two-step serum-free culture system supports development of human oocytes from primordial follicles in the presence of activin. *Hum Reprod.* 23(5): 1151-8.

Tilly JL & Sinclair DA (2013). Germline energetics, aging, and female infertility. *Cell Metab.* 17(6): 838-50.

Torrance C, Telfer E & Gosden RG (1989). Quantitative study of the development of isolated mouse pre-antral follicles in collagen gel culture. *J Reprod Fertil.* 87(1): 367-74.

Toyooka Y, Tsunekawa N, Takahashi Y, Matsui Y, Satoh M & Noce T (2000). Expression and intracellular localization of mouse Vasa-homologue protein during germ cell development. *Mech Dev.* 93(1-2): 139-49.

Trapphoff T, El Hajj N, Zechner U, Haaf T & Eichenlaub-Ritter U (2010). DNA integrity, growth pattern, spindle formation, chromosomal constitution and imprinting patterns of mouse oocytes from vitrified pre-antral follicles. *Hum Reprod.* 25(12): 3025-42.

Tripurani SK, Lee KB, Wang L, Wee G, Smith GW, Lee YS, Latham KE & Yao J (2011). A novel functional role for the oocyte-specific transcription factor newborn ovary homeobox (NOBOX) during early embryonic development in cattle. *Endocrinology.* 152(3): 1013-23.

Tung JY, Rosen MP, Nelson LM, Turek PJ, Witte JS, Cramer DW, Cedars MI & Pera RA (2006). Variants in Deleted in AZoospermia-Like (DAZL) are correlated with reproductive parameters in men and women. *Hum Genet.* 118(6): 730-40.

Vainio S, Heikkila M, Kispert A, Chin N & Mcmahon AP (1999). Female development in mammals is regulated by Wnt-4 signalling. *Nature.* 397(6718): 405-9.

Vanacker J, Dolmans MM, Luyckx V, Donnez J & Amorim CA (2014). First transplantation of isolated murine follicles in alginate. *Regen Med.* 9(5): 609-19.

Vanacker J, Luyckx V, Dolmans MM, Des Rieux A, Jaeger J, Van Langendonckt A, Donnez J & Amorim CA (2012). Transplantation of an alginate-matrigel matrix containing isolated ovarian cells: first step in developing a biodegradable scaffold to transplant isolated preantral follicles and ovarian cells. *Biomaterials.* 33(26): 6079-85.

Vermande-Van Eck GJ (1956). Neo-Ovogenesis in the Adult Monkey - Consequences of Atresia of Ovocytes. *Anatomical Record.* 125(2): 207-224.

Villano CM & White LA (2006). Expression of the helix-loop-helix protein inhibitor of DNA binding-1 (ID-1) is activated by all-trans retinoic acid in normal human keratinocytes. *Toxicol Appl Pharmacol.* 214(3): 219-29.

Vincent SD, Dunn NR, Sciammas R, Shapiro-Shalef M, Davis MM, Calame K, Bikoff EK & Robertson EJ (2005). The zinc finger transcriptional repressor Blimp1/Prdm1 is dispensable for early axis formation but is required for specification of primordial germ cells in the mouse. *Development.* 132(6): 1315-25.

Virant-Klun I, Skutella T, Hren M, Gruden K, Cvjeticanin B, Vogler A & Sinkovec J (2013). Isolation of small SSEA-4-positive putative stem cells from the ovarian surface epithelium of adult human ovaries by two different methods. *Biomed Res Int.* 2013: 690415.

Virant-Klun I, Zech N, Rozman P, Vogler A, Cvjeticanin B, Klemenc P, Malicev E & Meden-Vrtovec H (2008). Putative stem cells with an embryonic character isolated from the ovarian surface epithelium of women with no naturally present follicles and oocytes. *Differentiation.* 76(8): 843-856.

Viswanathan SR, Daley GQ & Gregory RI (2008). Selective blockade of microRNA processing by Lin28. *Science.* 320(5872): 97-100.

Vogel G (2012). REPRODUCTIVE BIOLOGY Potential Egg Stem Cells Reignite Debate. *Science.* 335(6072): 1029-1030.

Waldeyer W (1870). *Eierstock und Ei*, Leipzig, Engelmann.

Walker ML & Herndon JG (2008). Menopause in nonhuman primates? *Biol Reprod.* 79(3): 398-406.

Wallace WH, Anderson RA & Irvine DS (2005). Fertility preservation for young patients with cancer: who is at risk and what can be offered? *Lancet Oncol.* 6(4): 209-18.

Wallace WHB & Kelsey TW (2010). Human Ovarian Reserve from Conception to the Menopause. *Plos One.* 5(1).

Wang H, Jiang M, Bi H, Chen X, He L, Li X & Wu J (2014). Conversion of female germline stem cells from neonatal and prepubertal mice into pluripotent stem cells. *J Mol Cell Biol.* 6(2): 164-71.

Wells DN, Misica PM, Tervit HR & Vivanco WH (1998). Adult somatic cell nuclear transfer is used to preserve the last surviving cow of the Enderby Island cattle breed. *Reprod Fertil Dev.* 10(4): 369-78.

West ER, Shea LD & Woodruff TK (2007). Engineering the follicle microenvironment. *Semin Reprod Med.* 25(4): 287-99.

White YA, Woods DC, Takai Y, Ishihara O, Seki H & Tilly JL (2012). Oocyte formation by mitotically active germ cells purified from ovaries of reproductive-age women. *Nat Med.* 18(3): 413-21.

Wolff E (2016). Orthotopic Transplantation of Ovarian-Derived Stem Cells Results in Mature Oocytes That Can Fertilize and Undergo Embryo Development in Non-Human Primates F15 [Abstract]. *SSR Annual Meeting* 100.

Wolff E, Libfraind L, Weitzel R, Tilly J, Decherney A & Tisdale J (2013). Oogonial stem cells generate mature oocytes in an autologous Rhesus macaque transplantation model O-133 [Abstract]. *Fertil Steril.* 100(3): S40.

Wolff E, Libfraind L, Weitzel R, Woods D, Feng Y, Tilly J, Decherney A & Tisdale J (2014). Oogonial Stem Cells Generate Mature Oocytes in an Autologous Rhesus Macaque Transplantation Model O-126 [Abstract]. *Reprod Sci.* 21(3 (Supplement)): 119A.

Wongtrakoongate P, Jones M, Gokhale PJ & Andrews PW (2013). STELLA facilitates differentiation of germ cell and endodermal lineages of human embryonic stem cells. *PLoS One.* 8(2): e56893.

Woods DC & Tilly JL (2012). The next (re)generation of ovarian biology and fertility in women: is current science tomorrow's practice? *Fertil Steril.* 98(1): 3-10.

Woods DC & Tilly JL (2013). Isolation, characterization and propagation of mitotically active germ cells from adult mouse and human ovaries. *Nature Protocols.* 8: 966-88.

Woods DC & Tilly JL (2015). Woods and Tilly reply. *Nat Med.* 21(10): 1118-21.

Woods DC, White YA & Tilly JL (2013). Purification of oogonial stem cells from adult mouse and human ovaries: an assessment of the literature and a view toward the future. *Reprod Sci.* 20(1): 7-15.

Wycherley G, Downey D, Kane MT & Hynes AC (2004). A novel follicle culture system markedly increases follicle volume, cell number and oestradiol secretion. *Reproduction.* 127(6): 669-77.

Xie T & Spradling AC (2000). A niche maintaining germ line stem cells in the Drosophila ovary. *Science.* 290(5490): 328-30.

Xie W, Wang H & Wu J (2014). Similar morphological and molecular signatures shared by female and male germline stem cells. *Sci Rep.* 4: 5580.

Xiong J, Lu Z, Wu M, Zhang J, Cheng J, Luo A, Shen W, Fang L, Zhou S & Wang S (2015). Intraovarian Transplantation of Female Germline Stem Cells Rescue Ovarian Function in Chemotherapy-Injured Ovaries. *PLoS One.* 10(10): e0139824.

Yamaguchi S, Kimura H, Tada M, Nakatsuji N & Tada T (2005). Nanog expression in mouse germ cell development. *Gene Expr Patterns.* 5(5): 639-46.

Yamaguchi S, Kurimoto K, Yabuta Y, Sasaki H, Nakatsuji N, Saitou M & Tada T (2009). Conditional knockdown of Nanog induces apoptotic cell death in mouse migrating primordial germ cells. *Development.* 136(23): 4011-20.

Yang J, Li X, Li Y, Southwood M, Ye L, Long L, Al-Lamki RS & Morrell NW (2013). Id proteins are critical downstream effectors of BMP signaling in human pulmonary arterial smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol.* 305(4): L312-21.

Yang MY & Fortune JE (2008). The capacity of primordial follicles in fetal bovine ovaries to initiate growth in vitro develops during mid-gestation and is associated with meiotic arrest of oocytes. *Biol Reprod.* 78(6): 1153-61.

Ying Y, Liu XM, Marble A, Lawson KA & Zhao GQ (2000). Requirement of Bmp8b for the generation of primordial germ cells in the mouse. *Mol Endocrinol.* 14(7): 1053-63.

Ying Y & Zhao GQ (2001). Cooperation of endoderm-derived BMP2 and extraembryonic ectoderm-derived BMP4 in primordial germ cell generation in the mouse. *Dev Biol.* 232(2): 484-92.

Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin, Ii & Thomson JA (2007). Induced pluripotent stem cell lines derived from human somatic cells. *Science.* 318(5858): 1917-20.

Yuan J, Zhang D, Wang L, Liu M, Mao J, Yin Y, Ye X, Liu N, Han J, Gao Y, Cheng T, Keefe DL & Liu L (2013). No evidence for neo-oogenesis may link to ovarian senescence in adult monkey. *Stem Cells.* 31(11): 2538-50.

Yuan L, Liu JG, Hoja MR, Wilbertz J, Nordqvist K & Hoog C (2002). Female germ cell aneuploidy and embryo death in mice lacking the meiosis-specific protein SCP3. *Science.* 296(5570): 1115-8.

Zarate-Garcia L, Lane SI, Merriman JA & Jones KT (2016). FACS-sorted putative oogonial stem cells from the ovary are neither DDX4-positive nor germ cells. *Sci Rep.* 6:27991).

Zhang D, Fouad H, Zoma WD, Salama SA, Wentz MJ & Al-Hendy A (2008). Expression of stem and germ cell markers within nonfollicle structures in adult mouse ovary. *Reprod Sci.* 15(2): 139-46.

Zhang H, Liu L, Li X, Busayavalasa K, Shen Y, Hovatta O, Gustafsson JA & Liu K (2014). Life-long in vivo cell-lineage tracing shows that no oogenesis originates from putative germline stem cells in adult mice. *Proc Natl Acad Sci U S A.* 111(50): 17983-8.

Zhang H, Panula S, Petropoulos S, Edsgard D, Busayavalasa K, Liu L, Li X, Risal S, Shen Y, Shao J, Liu M, Li S, Zhang D, Zhang X, Gerner RR, Sheikhi M, Damdimopoulou P, Sandberg R, Douagi I, Gustafsson JA, Liu L, Lanner F, Hovatta O & Liu K (2015). Adult human and mouse ovaries lack DDX4-expressing functional oogonial stem cells. *Nat Med.* 21(10): 1116-8.

Zhang H, Zheng W, Shen Y, Adhikari D, Ueno H & Liu K (2012). Experimental evidence showing that no mitotically active female germline progenitors exist in postnatal mouse ovaries. *Proc Natl Acad Sci U S A.* 109(31): 12580-5.

Zhang Y, Yang Z, Yang Y, Wang S, Shi L, Xie W, Sun K, Zou K, Wang L, Xiong J, Xiang J & Wu J (2011). Production of transgenic mice by random recombination of targeted genes in female germline stem cells. *J Mol Cell Biol.* 3(2): 132-41.

Zhao R, Xuan Y, Li X & Xi R (2008). Age-related changes of germline stem cell activity, niche signaling activity and egg production in Drosophila. *Aging Cell.* 7(3): 344-54.

Zhou L, Wang L, Kang JX, Xie W, Li X, Wu C, Xu B & Wu J (2014). Production of fat-1 transgenic rats using a post-natal female germline stem cell line. *Mol Hum Reprod.* 20(3): 271-81.

Zou K, Hou L, Sun K, Xie W & Wu J (2011). Improved efficiency of female germline stem cell purification using fragilis-based magnetic bead sorting. *Stem Cells Dev.* 20(12): 2197-204.

Zou K, Yuan Z, Yang Z, Luo H, Sun K, Zhou L, Xiang J, Shi L, Yu Q, Zhang Y, Hou R & Wu J (2009). Production of offspring from a germline stem cell line derived from neonatal ovaries. *Nat Cell Biol.* 11(5): 631-6.

Zuckerman S (1951). The number of oocytes in the mature ovary. *Rec Prog Horm Res.* 6: 63–108.

Appendix 1

Manufacturers' Details

Manufacturer	Location	Country
Abcam	Cambridge	UK
AbD Serotec®	Oxford	UK
Acumedic®	London	UK
Agilent Technologies	Stockport	UK
BD Biosciences	Oxford	UK
Beckman Coulter	High Wycombe	UK
Bemis	Wisconsin	USA
Bioline	London	UK
Bio-Rad	Hemel Hempstead	UK
Cambridge Bioscience	Cambridge	UK
CellPath	Powys	UK
Cell Signaling Technology	Leiden	The Netherlands
Charles River	Ormiston	UK
Clontech Laboratories, Inc.	California	USA
Corning	Flintshire	UK
Dako	Cambridgeshire	UK
Eppendorf	Stevenage	UK
Enzo Life Sciences	Exeter	UK
Fisher Scientific	Loughborough	UK
GE Healthcare Life Sciences	Amersham	UK
GraphPad Software Inc.	California	USA
Greiner Bio-One	Gloucestershire	UK
Hamamatsu Photonics UK Ltd.	Welwyn Garden City	UK
Instant Pot Company	Ottawa	Canada

Integrated DNA Technologies	Leuven	Belgium
Jackson ImmunoResearch	Newmarket	UK
Leica	Milton Keynes	UK
Li-Cor Biotechnology	Cambridge	UK
Life Technologies	Paisley	UK
Lymphotec Inc.	Tokyo	Japan
Merck Millipore	Watford	UK
Microsoft	Washington	USA
Miltenyi Biotec	Bisley	UK
Mirus	Wisconsin	USA
National Institutes of Health	Maryland	USA
Olympus	Southend-on-Sea	UK
OriGene	Maryland	USA
Partec	Kent	UK
Perkin Elmer	Buckinghamshire	UK
Pharmacia and Upjohn Company	Michigan	USA
Photometrics	Arizona	USA
Qiagen	Manchester	UK
R&D Systems	Abingdon	UK
Roche	Burgess Hill	UK
Rockland	Reading	UK
Santa Cruz Biotechnology, Inc.	Dallas	USA
Sartorius Mechatronics Ltd.	Epsom	UK
Sigma-Aldrich	Gillingham	UK
Spherotech	Illinois	USA
Syngene	Cambridge	UK

Thermo Scientific	Paisley	UK
Vector Laboratories	Peterborough	UK
VHBio Ltd.	Gateshead	UK
VWR International	Lutterworth	UK
World Precision Instruments Ltd.	Hitchin	UK
Worthington Biochemical Company	Reading	UK
Zeiss	Cambridge	UK

Appendix 2

Published Papers

Maturitas xxx (2013) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Maturitas

journal homepage: www.elsevier.com/locate/maturitas

Review

Ovarian stem cells—Potential roles in infertility treatment and fertility preservation

Cheryl E. Dunlop[a], Evelyn E. Telfer[b], Richard A. Anderson[a,*]

[a] MRC Centre for Reproductive Health, University of Edinburgh, Queens Medical Research Institute, Edinburgh, Scotland, UK
[b] Centre for Integrative Physiology, University of Edinburgh, George Square, Edinburgh, Scotland, UK

ARTICLE INFO

Article history:
Received 15 March 2013
Received in revised form 16 April 2013
Accepted 25 April 2013
Available online xxx

Keywords:
Ovarian stem cell
Infertility
Fertility preservation
IVF
Premature ovarian insufficiency

ABSTRACT

One of the principal beliefs in reproductive biology is that women have a finite ovarian reserve, which is fixed from the time they are born. This theory has been questioned recently by the discovery of ovarian stem cells which are purported to have the ability to form new oocytes under specific conditions post-natally. Almost a decade after their discovery, ovarian, or oogonial, stem cells (OSCs) have been isolated in mice and humans but remain the subject of much debate. Studies in mice have shown that these cells can be cultured to a mature oocyte stage in vitro, and when injected into germ-cell depleted ovary they can form follicles and have resulted in the birth of healthy offspring. There are few data from human OSCs but this finding would open the door to novel fertility preservation strategies for women with both age-related and premature ovarian insufficiency (POI). As the number of girls and young women surviving cancer increases worldwide, POI secondary to gonadotoxic treatments, such as chemotherapy, is becoming more common. The ideal fertility preservation approach would prevent delays in commencing life-saving treatment and avoid transplanting malignant cells back into a woman after treatment: OSCs may offer one route to achieving this. This review summarises our current understanding of OSCs and discusses their potential clinical application in infertility treatment and fertility preservation.

Contents

1. Introduction

The dogma that female mammals are born with all of the oocytes they will ever possess has its foundations in a paper from Sir Solomon Zuckerman published in 1951 [1]. Simply put, Zuckerman failed to find any experimental evidence available at that time that he felt was inconsistent with an earlier hypothesis [2] that germ cell production in female mammals ceases prior to birth (reviewed by Zuckerman) [3]. This paper and its main conclusion profoundly affected the subsequent interpretation of experimental and clinical observations relating to ovarian development, function and failure for the next 50 years. A paper published by Jonathan Tilly's laboratory in 2004 reignited this debate by reporting the presence of a

* Corresponding author at: MRC Centre for Reproductive Health, University of Edinburgh, Queens Medical Research Institute, 47 Little France Crescent, Edinburgh EH16 4TJ, UK. Tel.: +44 0131 2426386; fax: +44 0131 2426441.
E-mail address: Richard.anderson@ed.ac.uk (R.A. Anderson).

0378-5122/$ – see front matter © 2013 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.maturitas.2013.04.017

population of mitotically active germline stem cells (GSCs) in the mouse ovary which, the authors postulated, maintain oocyte and follicle production in the ovary after birth [4]. The finding of GSCs, or oogonial stem cells (OSCs) as they are now more commonly known, has generated a lively debate in the field over the last decade as it is in direct opposition to the dogma that female mammals have a non-renewable oocyte reserve from birth. This debate has been perceived as representing two clearly opposing viewpoints with no common ground (reviewed by Powell) [5], but there is the possibility that both views can co-exist, with the formation of a population of oocytes at birth that is the main contributor to ovarian function and fertility and subject to little, if any, renewal and the existence of OSCs in adult ovaries that can only be activated under specific circumstances. It is impossible to prove the absence of any given cell in a tissue but the debate cannot be resolved until the presence and function of OSCs within adult ovaries can be unequivocally demonstrated.

Regardless of the physiological significance of these cells what is undeniable are the possible clinical applications of OSCs in infertility and fertility preservation if their potential can be harnessed; this review will address the background to current understanding of OSCs, and provide a speculative discussion of their potential clinical applications. If human OSCs can be grown into fully functional oocytes, can this be harnessed to address the age-related decline in oocyte quality? Could girls and young women about to undergo gonadotoxic therapy, e.g. for cancer, be able to cryopreserve some OSCs within their ovarian cortex prior to commencing treatment? Instead of concentrating on the finite number of primordial follicles within that ovarian tissue, it is conceivable that OSCs could subsequently be retrieved from this tissue and either cultured to form mature oocytes for use in in vitro fertilisation (IVF), or injected back into the woman's ovarian cortex for in vivo development. The number of new follicles that could be generated from OSCs could be much larger than the number of follicles in the stored ovarian tissue, and certainly much larger than the number of mature oocytes that a woman could store using the conventional approach of ovarian stimulation and aspiration of mature oocytes.

1.1. Identification and isolation of OSCs

Johnson et al. identified cells they considered OSCs whilst investigating follicular atresia in the mouse ovary [4]. They discovered that follicles were dying at a rate such that the ovary would be deplete of oocytes far earlier than is found in vivo. Analysis of the ovary revealed ovoid cells that both immunostained for a germ-cell specific marker (mouse vasa homologue or MVH, a germ-cell specific RNA helicase) and demonstrated incorporation of 5-bromodeoxyuridine (BrdU), indicative of proliferating cells. Furthermore, these cells expressed a meiosis-specific protein (synaptonemal complex protein 3, SCP3) required to initiate meiosis for the production of oocytes. In their final set of experiments, ovarian tissue from wild-type mice was transplanted onto the ovaries of mice which ubiquitously expressed green fluorescent protein (GFP). After 3–4 weeks, the wild-type ovary contained GFP-positive oocytes surrounded by wild-type granulosa cells, persuading the authors that OSCs from the GFP mouse had initiated folliculogenesis in the wild-type mouse and that they had discovered mitotically active OSCs that had the ability to form new oocytes after birth [4].

However, scepticism surrounded the idea of OSCs amongst reproductive biologists [6,7]. A key finding supporting claims that adult mouse ovaries retain the capacity for oogenesis came in a paper that reported that OSCs had been isolated and cultured from neonatal and adult mouse ovaries [8]. These cells, termed female germline stem cells (FGSC), were initially identified using the same criteria used by Johnson et al. [4] i.e., expression of MVH and BrdU

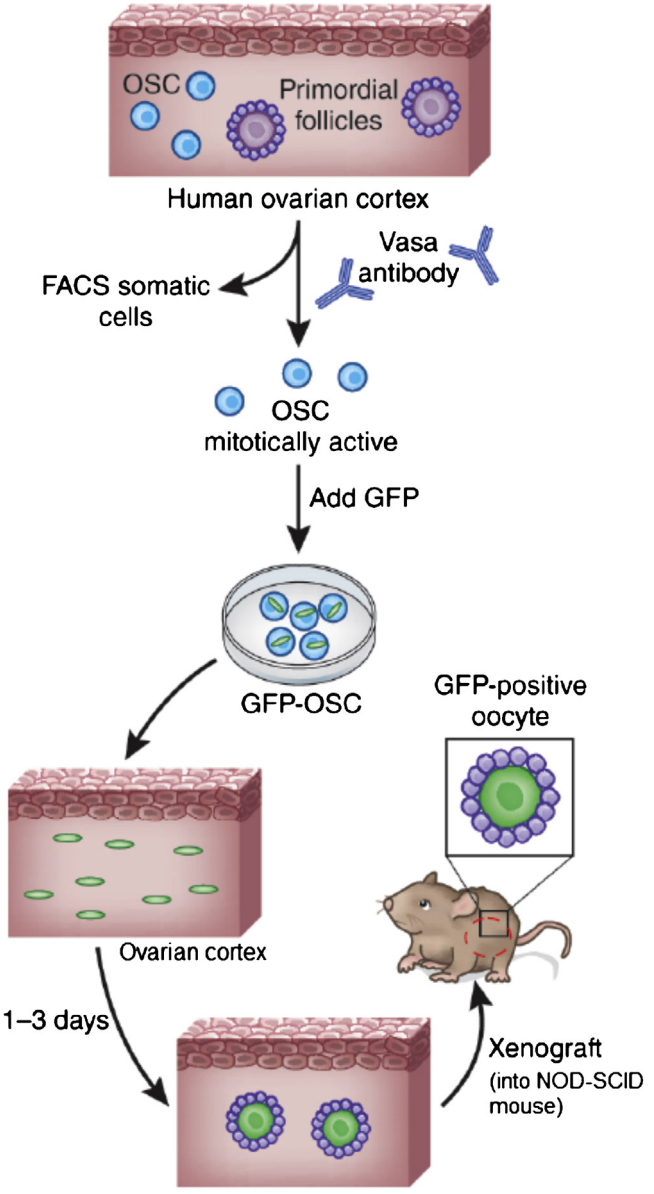


Fig. 1. White et al.'s method of isolating, purifying and culturing human OSCs using a xenograft. FACS: fluorescent-activated cell sorting.
From Telfer and Albertini [43].

incorporation. By employing a cell-sorting approach using an antibody against Ddx4 (DEAD box polypeptide 4, another name for MVH), the authors reported the ability to isolate and purify OSCs in mice. Furthermore, by transplanting GFP-positive OSCs into the ovaries of infertile mice, they were able to produce live GFP-positive offspring.

The main findings of this key study were developed further by White et al. [9] who not only managed to isolate human OSCs using DDX4 (the human orthologue of MVH, or VASA), but they were able to isolate, culture and form early follicle-like structures after injection of both mouse and human OSCs into ovarian tissue which was xenotransplanted into NOD-SCID (non-obese diabetic – severe combined immunodeficiency) mice to provide a suitable environment for early folliculogenesis (Fig. 1) [9].

Interestingly, and from an entirely separate line of evidence, the case for post-natal neo-oogenesis has been bolstered by a recent analysis of the accumulation of microsatellite mutations

Please cite this article in press as: Dunlop CE, et al. Ovarian stem cells—Potential roles in infertility treatment and fertility preservation. Maturitas (2013), http://dx.doi.org/10.1016/j.maturitas.2013.04.017

in the germline in female mice. There was a positive correlation between thus-determined oocyte 'depth' and mouse age, i.e. oocytes in older mice were found to have undergone more mitotic divisions than those in younger mice [10]. Although this may be in part explained by the "production-line" hypothesis, whereby oocytes are ovulated in the order in which they were formed [11], that explanation does not appear adequate for the degree of 'depth' identified, and the alternative explanation of post-natal de novo oogenesis could not be ruled out experimentally by the authors [12].

Of note, OSCs are not the only stem cells to have been shown to produce oocyte-like cells under the right conditions. Hayashi et al. recently published evidence that both embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) can be induced into primordial germ cell-like cells (PGCLCs) which, upon combination with gonadal somatic cells and transplantation into mice, can generate germinal vesicle-stage oocytes [13]. These oocytes can then be matured in vitro and, after IVF, can produce offspring which are themselves fertile. This study highlights the need to consider the appropriate stage of somatic cell support and so far has only been carried out in mice, but it offers an alternative model of germ cell development.

1.2. Controversy surrounding OSCs

The isolation of OSCs has been independently performed by only a small number of other groups to date [14–16] although full corroboration is as yet lacking. Many criticisms have been levelled at the experimental techniques utilised and the interpretation of the findings in these OSC experiments [7,17]. For example, mathematical modelling has been used to challenge Johnson et al.'s follicular atresia rate finding [18], although mathematical data supporting the theory of post-natal folliculogenesis in the mouse has also been produced [19]. Critics have been sceptical of the use of Ddx4/DDX4 as a cell surface marker in cell sorting as it has an intracytoplasmic localisation in oocytes [20]. However, it has been proposed that Ddx4/DDX4 has a transmembrane-spanning domain in OSCs, before becoming intracellular in more mature oocytes [9], although experimental evidence for this is still lacking. The argument for the existence of OSCs has been further strengthened by subsequent work reporting the isolation of OSCs in mice using alternative markers for cell sorting [14,21]. Wu's lab showed improved purification efficiency by employing interferon-inducible transmembrane protein 3 (Ifitm3, or fragilis), a widely accepted early germ cell-specific surface marker [21], whilst Pacchiarotti et al. used transgenic mice that expressed GFP under the control of another germline-specific marker, Oct-4 [14,22].

It has been questioned why these cells have gone undiscovered for so long [6]; however, it seems that OSCs are exceedingly rare, with White et al., reporting that they constitute a mere 0.014% of all cells in mouse ovaries [9]. They also seem to become rarer with increasing age, declining from 1 to 2% of cells in the neonatal mouse ovary to 0.05% in the adult [13]. The variation between values reported by different groups is likely to reflect the very different isolation methods used. This also perhaps explains the timespan between the initial purported discovery of these cells and their isolation from ovarian cortex. Furthermore, there are concerns about in vitro transformation given that the length of time it takes for these cells to establish in culture (10–12 weeks in the mouse and 4–8 weeks in humans) [9] is much greater than for the equivalent cells in the male [23]. Still, live offspring have been produced from such OSC cultures in mice [8] and early follicle-like structures have been generated in human tissue [9]. Despite these on-going disputes, the discovery of OSCs offers exciting new potential strategies for clinical application.

1.3. Clinical applications of OSCs in infertility

1.3.1. Age-related infertility

The age-related decline in female fertility is a central feature of human reproduction [24]. Whilst not the only aspect of concern, it is within the ovary and oocyte that most of this decline resides and will be of greater significance as pregnancy at advanced age becomes more prevalent. Currently the main strategy reproductive medicine has to offer is the use of oocyte donation: whilst undoubtedly successful, it is of huge consequence both for individual couples and for society [25]. 'Social' oocyte storage is increasingly prevalent, but even with multiple ovarian stimulation cycles the number of oocytes will be limited, and the financial costs high. Might OSCs have a role here? The isolation of OSCs from older women indicates the possibility, and the fact that they are capable of proliferation (i.e. they are pre-meiotic) means that the age-related aneuploidy risk might not exist, or at least be much less of an issue. OSCs also provide an unparalleled opportunity for the improved understanding of the basis of such clinically relevant aspects of human oocyte biology. Options for both the treatment and prevention of age-related fertility loss therefore exist at least in theory, but are critically dependant on understanding of whether, and why, neo-oogenesis ceases at the menopause.

1.3.2. Iatrogenic premature ovarian insufficiency

The survival rates from childhood cancer have increased significantly over the last 15–20 years [26], resulting in many young women suffering from premature ovarian insufficiency (POI) after being exposed to gonadotoxic treatment. At present, approaches available to women who want to attempt to preserve their fertility prior to gonadotoxic therapy include having oocytes or embryos cryopreserved after undergoing ovarian stimulation (and subsequent IVF with their partner's or donor's sperm in the case of embryo cryopreservation). Unfortunately, both of these approaches involve hormonal medication and lead to delays in commencing treatment for the patient's disease. The alternative option, which is the only approach appropriate for pre-pubertal girls [27,28], is cryopreservation of ovarian tissue with subsequent re-transplantation into the woman after cessation of her treatment. The first livebirth from this method was in 2004 when a woman with successfully treated Hodgkin's lymphoma underwent orthotopic autotransplantation of cryopreserved ovarian tissue [29] and there have been a number of livebirths reported since [30]. There have, however, been concerns surrounding this approach with certain diagnoses as there is a risk of reintroducing malignant cells within the transplant into the patient, particularly with haematological malignancies [27,31,32]. Consequently, an approach that avoids a delay in commencing life-saving treatment and employs a method to prevent transplanting cancer cells back into a patient would be preferable. OSCs may offer the potential to provide this approach.

To date, human OSCs have only been grown to early follicle-like structures in a xenotransplantation model [9], which is unacceptable in clinical use. However, the development of a multi-step culture system in cows and humans that supports folliculogenesis and oocyte growth from the primordial follicle stage means that there is the potential to produce mature oocytes from OSCs completely in vitro, if the correct somatic cell support is available [33,34] (Fig. 2). This serum-free culture model supports oocyte development in a shorter time frame compared with that seen in vivo, by culturing primordial follicles in small ovarian cortical strips to the pre-antral stage, before these pre-antral follicles are dissected out and cultured individually in activin-supplemented media [33,34]. Once the follicles reach the antral stage, the oocyte–granulosa cell complexes (OGCs) can be removed and placed on membranes for a final period of development prior to undergoing in vitro maturation (IVM) [35,36].

Please cite this article in press as: Dunlop CE, et al. Ovarian stem cells—Potential roles in infertility treatment and fertility preservation. Maturitas (2013), http://dx.doi.org/10.1016/j.maturitas.2013.04.017

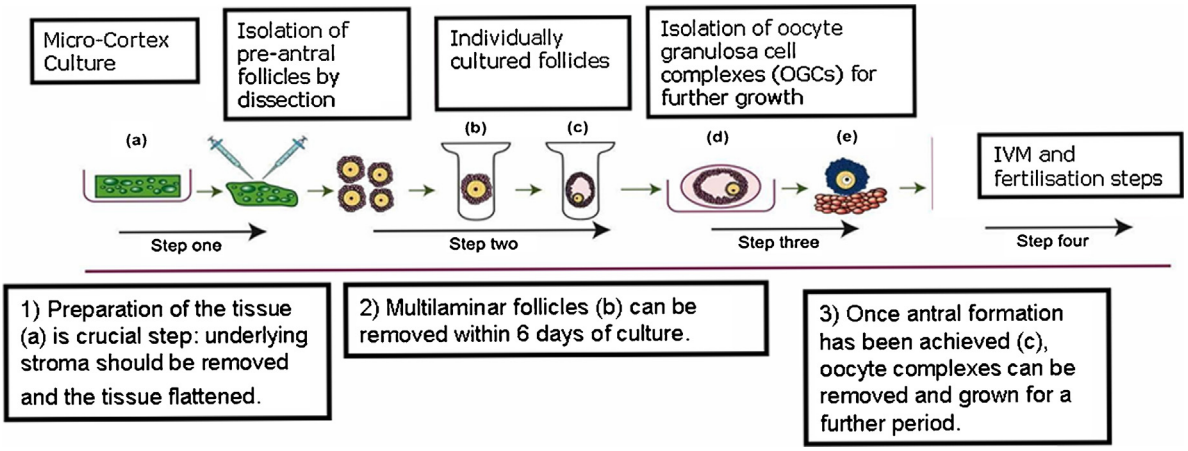


Fig. 2. A multi-step, serum-free culture system for in vitro human follicle development from the primordial follicle stage to isolation of oocytes for in vitro maturation (IVM). OGC: oocyte–granulosa cell complex.
From Telfer and McLaughlin [44].

Oocyte development and maturation is a multifaceted process and, as such, livebirths have only been achieved with follicles/oocytes grown from the primordial stage in mice [37,38]. In particular, there are concerns that in vitro oocyte culture and IVM may interfere with the complex genome imprinting stages and epigenetic mechanisms that are required for the development of a fully competent oocyte and subsequent embryo [39]. Animal studies have so far been reassuring; human studies will be a very considerable challenge.

With regard to human OSCs, if they can be transplanted into human ovarian cortex and the ensuing primordial follicles cultured in vitro in a serum-free multi-step system, any resultant mature, competent oocytes could be used in IVF. This opens the door to a novel fertility preservation approach that would be of use in those female patients who, for reasons of age or urgency for treatment, cannot have mature oocytes removed prior to gonadotoxic treatments that may render them infertile. Additionally, the expansion of OSC number in culture may allow for a considerably greater number of oocytes to be available to the women.

Another possible strategy is the injection of isolated OSCs into a patient's ovaries, where they, theoretically, could undergo neo-oogenesis in vivo and generate an entire population of hormone-secreting follicles as well as allowing a more physiological oocyte maturation. This may have the additional benefit of reversing the climateric symptoms and general health consequences associated with the menopause, at least temporarily. As with in vitro folliculogenesis, this remains a distant prospect at present.

1.3.3. Non-iatrogenic premature ovarian insufficiency

Although an increasing number of women with POI have an underlying iatrogenic cause [40], the aetiology of POI is heterogeneous, with the majority of cases being idiopathic [41]. Unless a family is aware of a hereditary genetic cause, women with non-iatrogenic POI often present too late for current fertility preservation methods to be feasible. At present, these women can either hope to spontaneously conceive (with an approximately 5% chance of spontaneously conceiving following diagnosis [41]), or opt to use donor oocytes and IVF. Hypothetically, OSCs could also be employed in the management of these women as it may be possible to isolate OSCs from these women's ovaries, despite their menopausal state. Indeed, OSCs have already been isolated in aged mouse ovaries and have been shown to undergo oogenesis when transplanted into a young mouse [42]. Again, these OSCs could be cultured as above and used in IVF.

Of note, White et al. have shown that OSCs can be isolated from cryopreserved, as well as fresh, human ovarian tissue [9]. This is encouraging from a fertility preservation point-of-view as it means that ovarian cortical tissue removed from girls or women can be safely stored until OSCs are required, thus allowing for technical advances in their isolation.

2. Conclusion

Few topics in the field of reproductive biology have led to as much animated discussion as the existence, or lack thereof, of oogonial stem cells. There is, however, increasing evidence that a rare population of cells with germline characteristics and proliferative capabilities can be retrieved from the ovaries of some species, including humans. What the purpose of these cells is under "normal" circumstances and what potential uses they have is still unclear; recent progress has been promising but their potential therapeutic use remains distant. Their existence also challenges current concepts as to the basis of the menopause: if there remains a potential oocyte source in the ovaries of postmenopausal women, what causes their development into follicles to stop? Although there is a very long way to go before the findings discussed here may have a clinical application in human infertility and fertility preservation, it seems likely that novel treatments may result from oogonial stem cell research in the future.

Contributors

Dr. Cheryl E. Dunlop contributed in writing of ms, approval of final version. Dr. Evelyn E. Telfer contributed in writing of ms, approval of final version. Professor Richard A. Anderson contributed in writing of ms, approval of final version.

Competing interest

None.

Provenance and peer review

Commissioned and externally peer reviewed.

Acknowledgements

The authors' research in this field is supported by MRC grants G1100357 and G0901839.

References

[1] Zuckerman S. The number of oocytes in the mature ovary. Recent Progress in Hormone Research 1951;6:63–108.
[2] Waldeyer W. Eierstock und Ei. Leipzig: Engelmann; 1870.
[3] Zuckerman S. Beyond the Ivory Tower: the frontiers of public and private science. New York: Taplinger Pub. Co.; 1971.
[4] Johnson J, Canning J, Kaneko T, Pru JK, Tilly JL. Germline stem cells and follicular renewal in the postnatal mammalian ovary. Nature 2004;428(Marvh (6979)):145–50.
[5] Powell K. Going against the grain. PLoS Biology 2007;5(December (12)):e338.
[6] Telfer EE, Gosden RG, Byskov AG, et al. On regenerating the ovary and generating controversy. Cell 2005;122(September (6)):821–2.
[7] Byskov AG, Faddy MJ, Lemmen JG, Andersen CY. Eggs forever? Differentiation 2005;73(December (9/10)):438–46.
[8] Zou K, Yuan Z, Yang Z, et al. Production of offspring from a germline stem cell line derived from neonatal ovaries. Nature Cell Biology 2009;11(May (5)):631–6.
[9] White YA, Woods DC, Takai Y, Ishihara O, Seki H, Tilly JL. Oocyte formation by mitotically active germ cells purified from ovaries of reproductive-age women. Nature Medicine 2012;18(March (3)):413–21.
[10] Reizel Y, Itzkovitz S, Adar R, et al. Cell lineage analysis of the mammalian female germline. PLoS Genetics 2012;8(2):e1002477.
[11] Henderson SA, Edwards RG. Chiasma frequency and maternal age in mammals. Nature 1968;218(April (5136)):22–8.
[12] Woods DC, Telfer EE, Tilly JL. Oocyte family trees: old branches or new stems? PLoS Genetics 2012;8(7):e1002848.
[13] Hayashi K, Ogushi S, Kurimoto K, Shimamoto S, Ohta H, Saitou M. Offspring from oocytes derived from in vitro primordial germ cell-like cells in mice. Science 2012;338(November (6109)):971–5.
[14] Pacchiarotti J, Maki C, Ramos T, et al. Differentiation potential of germ line stem cells derived from the postnatal mouse ovary. Differentiation 2010;79(March (3)):159–70.
[15] Hu Y, Bai Y, Chu Z, et al. GSK3 inhibitor-BIO regulates proliferation of female germline stem cells from the postnatal mouse ovary. Cell Proliferation 2012;45(August (4)):287–98.
[16] Simon C. Somatic stem cells and tissue engineering shed light on unsolved clinical issues in reproductive medicine: in stem cells we trust. Fertility and Sterility 2012;98(July (1)):1–2.
[17] Notarianni E. Reinterpretation of evidence advanced for neo-oogenesis in mammals, in terms of a finite oocyte reserve. Journal of Ovarian Research 2011;4(1):1.
[18] Bristol-Gould SK, Kreeger PK, Selkirk CG, et al. Fate of the initial follicle pool: empirical and mathematical evidence supporting its sufficiency for adult fertility. Developmental Biology 2006;298(October (1)):149–54.
[19] Kerr JB, Duckett R, Myers M, Britt KL, Mladenovska T, Findlay JK. Quantification of healthy follicles in the neonatal and adult mouse ovary: evidence for maintenance of primordial follicle supply. Reproduction 2006;132(July (1)):95–109.
[20] Castrillon DH, Quade BJ, Wang TY, Quigley C, Crum CP. The human VASA gene is specifically expressed in the germ cell lineage. Proceedings of the National Academy of Sciences of the United States of America 2000;97(August (17)):9585–90.
[21] Zou K, Hou L, Sun K, Xie W, Wu J. Improved efficiency of female germline stem cell purification using fragilis-based magnetic bead sorting. Stem Cells and Development 2011;20(December (12)):2197–204.
[22] Okamura D, Tokitake Y, Niwa H, Matsui Y. Requirement of Oct3/4 function for germ cell specification. Developmental Biology 2008;317(May (2)):576–84.
[23] Oatley J, Hunt PA. Of mice and (wo)men: purified oogonial stem cells from mouse and human ovaries. Biology of Reproduction 2012;86(June (6)):196.
[24] Nelson SM, Telfer EE, Anderson RA. The ageing ovary and uterus: new biological insights. Human Reproduction Update 2013;19(January–February (1)):67–83.
[25] Donchin A. Reproductive tourism and the quest for global gender justice. Bioethics 2010;24(September (7)):323–32.
[26] Magnani C, Pastore G, Coebergh JW, Viscomi S, Spix C, Steliarova-Foucher E. Trends in survival after childhood cancer in Europe, 1978–1997: report from the Automated Childhood Cancer Information System project (ACCIS). European Journal of Cancer 2006;42(September (13)):1981–2005.
[27] Anderson RA, Wallace WH. Fertility preservation in girls and young women. Clinical Endocrinology (Oxford) 2011;75(October (4)):409–19.
[28] Wallace WH, Anderson RA, Irvine DS. Fertility preservation for young patients with cancer: who is at risk and what can be offered? Lancet Oncology 2005;6(April (4)):209–18.
[29] Donnez J, Dolmans MM, Demylle D, et al. Livebirth after orthotopic transplantation of cryopreserved ovarian tissue. Lancet 2004;364(October (9443)):1405–10.
[30] Donnez J, Silber S, Andersen CY, et al. Children born after autotransplantation of cryopreserved ovarian tissue. A review of 13 live births. Annals of Medicine 2011;43(6):437–50.
[31] Dolmans MM, Marinescu C, Saussoy P, Van Langendonckt A, Amorim C, Donnez J. Reimplantation of cryopreserved ovarian tissue from patients with acute lymphoblastic leukemia is potentially unsafe. Blood 2010;116(October (16)):2908–14.
[32] Rosendahl M, Andersen MT, Ralfkiaer E, Kjeldsen L, Andersen MK, Andersen CY. Evidence of residual disease in cryopreserved ovarian cortex from female patients with leukemia. Fertility and Sterility 2010;94(November (6)):2186–90.
[33] Telfer EE, McLaughlin M, Ding C, Thong KJ. A two-step serum-free culture system supports development of human oocytes from primordial follicles in the presence of activin. Human Reproduction 2008;23(May (5)):1151–8.
[34] McLaughlin M, Telfer EE. Oocyte development in bovine primordial follicles is promoted by activin and FSH within a two-step serum-free culture system. Reproduction 2010;139(June (6)):971–8.
[35] Telfer EE, McLaughlin M. Natural history of the mammalian oocyte. Reproductive Biomedicine Online 2007;15(September (3)):288–95.
[36] Smitz J, Dolmans MM, Donnez J, et al. Current achievements and future research directions in ovarian tissue culture, in vitro follicle development and transplantation: implications for fertility preservation. Human Reproduction Update 2010;16(July–August (4)):395–414.
[37] Eppig JJ, O'Brien MJ. Development in vitro of mouse oocytes from primordial follicles. Biology of Reproduction 1996;54(January (1)):197–207.
[38] O'Brien MJ, Pendola JK, Eppig JJ. A revised protocol for in vitro development of mouse oocytes from primordial follicles dramatically improves their developmental competence. Biology of Reproduction 2003;68(May (5)):1682–6.
[39] Anckaert E, De Rycke M, Smitz J. Culture of oocytes and risk of imprinting defects. Human Reproduction Update 2013;19(January–February (1)):52–66.
[40] Panay N, Kalu E. Management of premature ovarian failure. Best Practice & Research: Clinical Obstetrics & Gynaecology 2009;23(February (1)):129–40.
[41] Goswami D, Conway GS. Premature ovarian failure. Hormone Research 2007;68(4):196–202.
[42] Niikura Y, Niikura T, Tilly JL. Aged mouse ovaries possess rare premeiotic germ cells that can generate oocytes following transplantation into a young host environment. Aging (Albany, NY) 2009;1(December (12)):971–8.
[43] Telfer EE, Albertini DF. The quest for human ovarian stem cells. Nature Medicine 2012;18(March (3)):353–4.
[44] Telfer EE, McLaughlin M. Strategies to support human oocyte development in vitro. The International Journal of Developmental Biology 2012;56(November):901–7.

Please cite this article in press as: Dunlop CE, et al. Ovarian stem cells—Potential roles in infertility treatment and fertility preservation. Maturitas (2013), http://dx.doi.org/10.1016/j.maturitas.2013.04.017

stem cell research&therapy

REVIEW

Ovarian germline stem cells

Cheryl E Dunlop[1], Evelyn E Telfer[2] and Richard A Anderson[1*]

Abstract

It has long been established that germline stem cells (GSCs) are responsible for lifelong gametogenesis in males, and some female invertebrates (for example, *Drosophila*) and lower vertebrates (for example, teleost fish and some prosimians) also appear to rely on GSCs to replenish their oocyte reserve in adulthood. However, the presence of such cells in the majority of female mammals is controversial, and the idea of a fixed ovarian reserve determined at birth is the prevailing belief among reproductive biologists. However, accumulating evidence demonstrates the isolation and culture of putative GSCs from the ovaries of adult mice and humans. Live offspring have been reportedly produced from the culture of adult mouse GSCs, and human GSCs formed primordial follicles using a mouse xenograft model. If GSCs were present in adult female ovaries, it could be postulated that the occurrence of menopause is not due to the exhaustion of a fixed supply of oocytes but instead is a result of GSC and somatic cell aging. Alternatively, they may be benign under normal physiological conditions. If their existence were confirmed, female GSCs could have many potential applications in both basic science and clinical therapies. GSCs not only may provide a valuable model for germ cell development and maturation but may have a role in the field of fertility preservation, with women potentially being able to store GSCs or GSC-derived oocytes from their own ovaries prior to infertility-inducing treatments. Essential future work in this field will include further independent corroboration of the existence of GSCs in female mammals and the demonstration of the production of mature competent oocytes from GSCs cultured entirely *in vitro*.

Introduction

Germline stem cells (GSCs) are a unique cell population committed to producing gametes for the propagation of the species. The concept of a GSC most likely originates from Regaud [1,2], whose work on spermatogenesis was published over a century ago. He postulated that, in order for sperm production to occur, a population of self-renewing cells must be present in the testis which could produce differentiated progeny. It is now well established that these cells, now known as spermatogonial stem cells, contribute to spermatogenesis in adulthood in the males of all species studied [3]. Research on the existence of a female counterpart, an ovarian GSC that is able to undergo postnatal *neo*-oogenesis and thus contribute to oocyte production in adulthood, has revealed a more complicated picture. Although female GSCs (fGSCs) appear to have a role in oogenesis throughout reproductive life in some non-mammalian species, these examples appear to be relatively rare across the phyla of the animal kingdom [4], and the presence of fGSCs in mammals has been greatly debated. Indeed, the prevailing view is that female mammals are born with a finite stock of mature oocytes that become exhausted with aging, a hypothesis first suggested by the 19th century embryologist Waldeyer [5]. General opinion changed at the beginning of the 20th century when the prevailing belief was in favor of *neo*-oogenesis in adulthood [6] until an influential article by Zuckerman [7] in 1951 reported no evidence that new oocytes are formed once a female is born, and the idea of a fixed ovarian reserve in mammals has been a central dogma in the field since. However, since 2004, a growing number of researchers have found cause to question this doctrine. The debate was reignited with the proposition [8], and subsequent isolation [9-13], of purported fGSCs (also known as oogonial stem cells, or OSCs).

Critically, the physiological role of these cells *in vivo* in the adult mammalian ovary has yet to be determined. Development and maturation of an oocyte entail a complex and multifaceted process which has to be tightly regulated in order for the oocyte to be competent for fertilization. This includes bidirectional communication

* Correspondence: richard.anderson@ed.ac.uk
[1]MRC Centre for Reproductive Health, Queen's Medical Research Institute, The University of Edinburgh, 47 Little France Crescent, Edinburgh EH16 4TJ, UK
Full list of author information is available at the end of the article

between the oocyte and its surrounding somatic cells, precise timing of cessation and resumption of meiosis, and correct genomic imprinting (reviewed last year by Li and Albertini [14] and Anckaert and colleagues [15]). Imprinting involves epigenetic alterations of the parental alleles by DNA methylation and determines whether the maternal or paternal gene will be expressed in the embryo. Incorrect imprinting can lead to conditions such as Angelman and Prader-Willi syndromes. Therefore, future research involving the culture of oocytes derived from purported adult mammalian fGSCs will have to ensure that these processes are intact for these cells to be useful in clinical practice. This review will examine the existence of OSCs in various species, consider where research in the field is heading, and assess the therapeutic potential of such cells.

Ovarian germline stem cells in non-mammalian species and prosimian primates

There are several animals in which fGSCs actively replenish the ovarian reserve postnatally. fGSCs in 'lower' invertebrates have been extensively studied in the fruit fly, *Drosophila* [4]. In this species, a few primordial germ cells (PGCs) are effectively 'segregated' in a special germ cell niche at the tip of each ovariole (16 to 18 tubes that make up the ovary) prenatally [16]. The environment within this niche, in contrast to environments elsewhere in the ovary, prevents the PGCs from differentiating, and these undifferentiated cells subsequently become fGSCs [17]. Postnatally, this niche controls the division of fGSCs and the production of new oocytes, therefore providing a continuous supply of germ cells throughout reproductive life.

fGSCs have also been reported in teleost fish, including the medaka (*Oryzias latipes*) [18] and zebrafish (*Danio rerio*) [19]. As in *Drosophila*, medaka have a germ cell niche, called the germinal cradle, situated in the ovarian cords [18]. Within this area reside mitotic cells that have the characteristics of fGSCs and that continuously supply the ovary with new oocytes. Furthermore, zebrafish possess a distinct zone on the ovarian surface to which germ cells are confined, and this may also be analogous to the *Drosophila* germ cell niche [19], suggesting evolutionary conservation across animal phyla. Oogenesis throughout reproductive life may be necessary for the huge numbers of eggs produced during the fish and fly life span and appears more similar to spermatogenesis than the restrictive processes of oogenesis and associated follicle development in higher mammals.

Although prior to 2004 it was widely believed that the vast majority of adult mammals lack fGSCs, a few exceptions had been described. The adult ovaries of two members of the loris family, which are prosimians related to the lemur, have been reported to possess mitotically active germ cells located within 'nests' in the ovarian cortex [20-22]. It has not been proven, however, that these cells, found in a slow loris (*Nycticebus coucang*) and a slender loris (*Loris tardigradus lydekkerianus*), are actually capable of undergoing folliculogenesis and producing mature oocytes.

If fGSCs can be identified in such animals, why would they not be present in the ovaries of the vast majority of adult female mammals? Zuckerman himself was actually an advocate for *neo*-oogenesis until his convictions were changed by his extensive review of the literature [23], in which he stated: 'None of the experimental and quantitative evidence which we have considered thus supports the view that oogenesis occurs in the adult ovary, and much of it bears very clearly against the proposition' [7].

Lack of evidence is not definitive, and proving that a cell does not exist is difficult, especially if they are a scarce population. So what is the evidence for the existence of fGSCs in adult mammals?

Ovarian germline stem cells in mammals

The discovery of purported fGSCs in adult mice occurred during an investigation of oocyte atresia and its role in follicular dynamics, when an apparent mathematical anomaly was observed. Johnson and colleagues [8] reported that follicular atresia was occurring at a rate such that the adult mouse should exhaust her ovarian reserve well before the age that it in fact occurs. This implied that the follicle pool must be replenished in adulthood by *neo*-oogenesis in order to sustain the reproductive life of the mouse, and considering the germ cell dynamics model of Faddy and colleagues [24], the authors suggested that the adult mouse has to make 77 new primordial follicles a day. On further investigation, a rare population of mitotically active ovoid cells in the ovarian surface epithelium (OSE), which expressed the germ cell-specific protein mouse vasa homolog (MVH), was identified. Furthermore, when small pieces of wild-type ovarian cortex were transplanted onto the ovaries of transgenic mice that ubiquitously expressed green fluorescent protein (GFP) for 3 to 4 weeks, GFP-positive oocytes surrounded by wild-type somatic cells were found within the wild-type graft. These results persuaded the authors that new oocytes must continue to be produced throughout reproductive life in mice and that the proliferating cells in the OSE may be putative fGSCs and therefore the source of the ongoing oogenesis.

The article by Johnson and colleagues was met with widespread criticism, and subsequent work from the Tilly group, who suggested that the source of these fGSCs was the bone marrow and peripheral blood [25], was even more controversial [26,27]. However, Zou and colleagues [9] took a step forward when they reported

the isolation of fGSCs from adult mice. Using a magnetically activated cell sorting technique, the authors isolated putative fGSCs measuring 12 to 20 μm in diameter by using an antibody against either DDX4 (DEAD box polypeptide 4; also known as vasa or MVH) or IFITM3 (interferon-induced transmembrane protein 3; also known as fragilis) [9,11]. These cells expressed both pluripotency and germ cell markers, had a normal karyotype, and were maternally imprinted. Evidence of their capacity to undergo oogenesis was provided when GFP-expressing fGSCs were transplanted into sterilized mice, with GFP-positive offspring being produced. These findings in adult mice were supported by subsequent articles by Pacchiarotti and colleagues [10] and Hu and colleagues [12], who reported isolation of putative fGSCs by using different techniques, though with limited demonstration of oocyte-like competence. The first, and only, published evidence of the existence of these cells in humans was provided by the Tilly group in 2012 [13]. White and colleagues [13] developed a fluorescence-activated cell sorting protocol that consistently isolated fGSCs, which the authors named OSCs, from both adult mice and humans. Measuring 5 to 8 μm, the cells were smaller than those isolated by Zou and colleagues [9] but expressed similar germ cell markers. The reason these cells have not been detected in the past may be explained by the fact that White and colleagues [13] estimated that the OSC population makes up only 0.014 % ± 0.002 % of the mouse ovary. The authors noted spontaneous production of oocyte-like cells from fGSCs in *in vitro* culture (also observed by Pacchiarotti and colleagues [10]); these cells showed expression of oocyte-specific and meiotic markers. Finally, by injecting GFP-expressing fGSCs into non-GFP ovarian cortex and xenotransplanting the tissue into mice, the authors reported that primordial follicles comprising a GFP-positive oocyte and wild-type granulosa cells could be seen on removal of the graft.

In addition to these putative fGSCs, another population of ovarian stem cells that reportedly differentiate into oocytes has been isolated from the OSE [28-30]. These cells, named very small embryonic-like (VSEL) stem cells, are cultured from OSE scrapings, are smaller than the fGSCs discussed above, and differ in morphology from those reported by White and colleagues [13]. The cells express a number of stem cell markers, including SSEA-4, and spontaneously generate large, oocyte-like cells in culture. Interestingly, Parte and colleagues [29] also isolated a second putative ovarian stem cell population, slightly larger in size than the VSEL stem cells and perhaps more analogous to fGSCs. They postulated that the VSEL stem cells are, in fact, the precursors of these larger cells, which may be tissue-committed ovarian stem cells [29]. To date, VSEL stem cells have been reported in adult mice, rabbits, sheep, marmoset monkeys, and humans [28,29], including postmenopausal women and women with premature ovarian insufficiency [31]. VSEL stem cells from the OSE would appear to be distinct from fGSCs; however, the existence of VSEL stem cells, much like that of fGSCs, has also been controversial [32].

More recent evidence for the existence of mammalian fGSCs has been published by a Mexican group working with three species of phyllostomid bats [33]. The use of these species of bats is especially pertinent because they share some reproductive similarities with primates, both anatomically and with respect to ovulation patterns. For example, *Glossophaga soricina* are polyoestrous monovulates with menstrual cycles of 22 to 26 days, including a luteal phase and periodic endometrial shedding [34]. Antonio-Rubio and colleagues [33] demonstrated that the ovaries of *Artibeus jamaicensis*, *Glossophaga soricine*, and *Sturnira lilium* are polarized, with a medullary region containing developing follicles and a cortical region containing both primordial follicles and a population of cells which looked similar to germ cells histologically. These cells, when analyzed with immunofluorescence, expressed proliferation, pluripotency, and early germline markers, including phosphorylated histone H3, POU5F1, DDX4, and IFITM3, and were termed adult cortical germ cells (ACGCs). The authors thus postulated that ACGCs may be involved in adult *neo*-oogenesis in these species, although, as with the loris species mentioned previously, this was not demonstrated in this study.

In addition to this emergent body of proof, there is indirect evidence to support *neo*-oogenesis in adult female mammals. Work on rhesus monkey ovaries in the 1950s demonstrated findings similar to those of Johnson and colleagues [8] in the mouse, with the observed rates of follicular atresia predicting that monkey ovarian reserve should be depleted within 2 years [35]. The author calculated that the maximum life span of an oocyte was 2 years, and therefore the data suggested that the new oocytes must be continually produced throughout reproductive life. Mathematical modeling has provided conflicting data, and both Bristol-Gould and colleagues [36] and Wallace and Kelsey [37] found that the 'germline stem cell model' did not fit follicular kinetics data in either mice [36] or humans [37]. Conversely, Kerr and colleagues [38] have published data in support of postnatal oogenesis. Although they did not find evidence of GSCs, the authors demonstrated that the mean number of primordial follicles in mice did not decline between days 7 and 100 of age, leading them to surmise that there is a mechanism by which postnatal neo-folliculogenesis sustains the follicular pool.

Further indirect evidence has come from lineage tracing, although this has also provided conflicting evidence; some

data have refuted the fGSC hypothesis, and some have been unable to disprove that postnatal neo-oogenesis exists [39,40]. Lei and Spradling [39] have reported that primordial follicles are very stable, with no evidence of high rates of turnover, therefore suggesting that the pool is sufficient to sustain fertility without the requirement of fGSCs. In contrast, by examining the accumulation of microsatellite mutations in mice, Reizel and colleagues [40] found that oocyte 'depth' increased with age; in other words, the older the mouse, the more mitotic divisions the oocyte has undergone. If neo-oogenesis were not occurring postnatally, then depth should be stable throughout life and independent of any interventions; however, depth was also shown to increase after ovariectomy and this would indicate neo-oogenesis [41]. The 'production-line hypothesis' of Henderson and Edwards [42] may go some way to explaining this observation; however, the formation of new oocytes after birth is a possible alternative explanation. The findings of Lei and Spradling have also been refuted by Bhartiya and colleagues [43], who observed germ cell 'cysts' in adult mice and sheep which, the authors believe, reflect clonal expansion of stem cells within the ovary.

Germline stem cell aging

If ovaries *are* capable of producing new oocytes during adulthood, then the obvious question is: why do women go through menopause? It has traditionally been believed that women enter menopause when their finite supply of oocytes has been exhausted; however, if *neo*-oogenesis does indeed exist, then the rate of new oocyte production must lessen with age in order for menopause to occur. There may be two potential underlying mechanisms: failure of the fGSCs to form oocytes or failure of the somatic environment to support oocyte development (or both). It is possible that fGSCs, like many other cells, undergo an aging process and thus lose their capacity to regenerate and differentiate. In *Drosophila*, there is a reduction in oocyte production with age, associated with declining rates of fGSC division and increased apoptosis of developing oocytes [44]. Furthermore, an age-dependent deterioration in germ cell niche signaling may affect the ability of fGSCs to regenerate [44]. Several putative causative mechanisms for these age-related changes have been proposed, including a decrease in bone morphogenetic protein (BMP) production by the germ cell niche, a reduction in GSC-niche cell adhesion via E-cadherin, and an increase in harmful reactive oxygen species [45]. Manipulation of all of these factors has been shown to increase fGSC life span [45].

It is likely that mammalian aging can also be attributed, at least partly, to age-related stem cell senescence, and hematopoietic, neural, and muscle stem cell function all demonstrate a decline in function (reviewed in [46]). With regard to ovarian GSCs, Pacchiarotti and colleagues [10] found that the number of fGSCs they were able to isolate from mouse ovaries diminished with increasing age of the mouse. Furthermore, a study has reported the presence of putative fGSCs in aged mice that appear to undergo folliculogenesis only when transplanted back into a young mouse ovary, thus implying that the surrounding ovarian environment may have a role to play in the ability of fGSCs to sustain a woman's reproductive function [47]. Therefore, the idea that the existence of menopause renders *neo*-oogenesis impossible is not necessarily correct: the two phenomena may co-exist. However, the key demonstration that fGSCs contribute to the postnatal follicle pool and potentially to fertility in a physiological context has not been made. Although fGSCs may be isolatable from ovarian tissue and potentially able to form oocytes within follicles after various manipulations, this may occur only under experimental conditions and they may not have any relevance to the normal processes of ovarian function.

Basic science uses for germline stem cells

The potential uses for fGSCs are numerous, particularly in basic science but potentially even in clinical applications. With regard to the former, fGSCs provide an exciting prospect as a germ cell model in order to study the development and maturation of the oocyte. Park and colleagues [48] have used adult mouse-derived fGSCs to investigate the effect of BMP4. BMPs are a member of the transforming growth factor-beta family of growth factors with a critical role in PGC specification [49,50] and have been shown to act on germ cells within the developing human ovary [51]. Treatment of fGSCs with BMP4 increased both the rate of *in vitro* differentiation into oocyte-like structures and the expression of genes associated with the initiation of meiosis: muscle-segment homeobox 1 (Msx1), Msx2, and stimulated by retinoic acid gene 8 (Stra8) [48].

fGSCs have also been genetically manipulated to produce transgenic mice. Zhang and colleagues [52] transfected female adult mouse GSCs with recombinant viruses containing vectors for different genes, including GFP. When transplanted into sterilized mice and mated with wild-type male mice, offspring heterozygous for the transfected genes were produced. Using a liposome-mediated transfection, the same group was also able to create a knockout mouse to investigate the role of the gene *Oocyte-G1* [52]. The ability to produce transgenic animals in this way could be an excellent tool for reproductive biologists in the future.

Therapeutic uses for germline stem cells

There is no doubt that if fGSCs can be shown to develop into mature, competent, correctly imprinted oocytes

in vitro, they will have great clinical potential; however, owing to technical and regulatory issues, it may be a long time before this potential can be fulfilled. For example, in the UK, research into whether fGSC-derived oocytes would be capable of fertilization and development into a blastocyst would be possible only with the approval of the Human Fertilization and Embryology Authority. Nevertheless, fGSCs *may* have a role in both fertility preservation and the reversal of reproductive senescence. With regard the former, it is conceivable that fGSCs could be used as a fertility preservation strategy for women who require gonadotoxic treatment for cancer that may render them infertile. A sample of ovarian cortex could be taken prior to commencing treatment, and fGSCs could be isolated and cryopreserved for future use. The fGSCs, when required, could subsequently be injected back into a woman's ovaries where they could undergo *neo*-folliculogenesis, or they could be cultured *in vitro* in ovarian cortex to a mature oocyte stage and resultant oocytes used in *in vitro* fertilization (IVF). The benefits of this approach are twofold: firstly, taking ovarian cortex samples would not require life-saving treatment to be delayed in contrast to the ovarian superovulation regimens required for oocyte and embryo cryopreservation; secondly, many more new follicles and oocytes could be achieved from fGSCs than would be present in cryopreserved tissue or from ovarian stimulation.

Women with age-related infertility or premature ovarian insufficiency may also benefit from fGSCs. 'Social' oocyte storage is becoming increasingly sought by women who are anxious about how much longer their ovarian reserve will last. However, this is an expensive endeavor, is not without health risks, and may result in only a small number of oocytes being cryopreserved. As mentioned previously, putative fGSCs have been reported in aged mice [47]; therefore, it is not impossible that women who are perimenopausal, prematurely or not, may have a very small number of these cells residing in their ovaries. The prospect of these cells growing into oocytes in the aged stromal environment is less certain; however, they may be able to be used in IVF. The idea of 'reversing' the reproductive clock and thereby avoiding the detrimental health effects and climacteric symptoms of menopause is appealing to some; however, the aging ovarian milieu may also restrict the use of fGSCs to this end. In summary, such clinical applications are currently aspirational but worthy of further investigation.

Germline stem cells – the future

The field of reproductive biology remains very skeptical of the idea that female mammalian GSCs exist and particularly that they have any physiological role in normal ovarian function. Further demonstration of their isolation and *in vitro* characteristics from a range of species is needed as a first step. The potential for fGSCs to differentiate into daughter cells that become mature oocytes in an *in vivo* environment remains to be demonstrated. Given the apparent scarcity of fGSCs in the female mouse ovary, this may prove difficult to demonstrate. For those groups who have already isolated putative fGSCs, the essential next steps are investigating the conditions under which these cells will develop into oocytes that are capable of fertilization and thus exploring their potential as gametes. For fGSCs to be used in a clinical context, a complete *in vitro* culture system will need to be developed. In this regard, we are currently investigating whether fGSCs can be grown into a mature oocyte by using a multi-step serum-free culture system that we have already shown promotes healthy follicular growth in bovine and human ovarian cortex [53-55].

Conclusions

The reported existence of female mammalian GSCs has stimulated much interest among reproductive biologists, many of whom are yet to be convinced that these cells are a real entity. However, there are now a growing number of reports of their isolation and culture, and strides are being taken to investigate their *neo*-oogenesis capabilities. Whether these cells have a physiological role has yet to be determined, and concerns remain that isolated putative fGSCs have undergone *in vitro* transformation in order to form oocytes; yet if their potential can be harnessed, they may contribute greatly to our understanding of oocyte development and may be of important clinical relevance.

Abbreviations

ACGC: Adult cortical germ cell; BMP: Bone morphogenetic protein; DDX4: DEAD box polypeptide 4; fGSC: Female germline stem cell; GFP: Green fluorescent protein; GSC: Germline stem cell; IFITM3: Interferon-induced transmembrane protein 3; IVF: *in vitro* fertilization; MSX: Muscle-segment homeobox; MVH: Mouse vasa homolog; OSC: Oogonial stem cell; OSE: Ovarian surface epithelium; PGC: Primordial germ cell; VSEL: Very small embryonic-like.

Competing interests

The authors declare that they have no competing interests.

Acknowledgments

The authors' work in this field is supported by Medical Research Council grants G0901839 and G1100357.

Author details

[1]MRC Centre for Reproductive Health, Queen's Medical Research Institute, The University of Edinburgh, 47 Little France Crescent, Edinburgh EH16 4TJ, UK. [2]Institute of Cell Biology and Centre for Integrative Physiology, Hugh Robson Building, University of Edinburgh, George Square, Edinburgh EH8 9XD, UK.

Published: 18 Aug 2014

References

1. Regaud C: **Etudes sur la structure des tubes seminiferes et sur la spermatogenese chez les mammiferes [Studies on the structure of seminiferous tubules and on spermatogenesis in mammals]. Part 1.** *Archives d'Anatomie microscopiques et de Morphologie experimentale* 1901, **4:**101–156.
2. Regaud C: **Etudes sur la structure des tubes seminiferes et sur la spermatogenese chez les mammiferes [Studies on the structure of seminiferous tubules and on spermatogenesis in mammals] . Part 2.** *Archives d'Anatomie microscopiques et de Morphologie experimentale* 1901, **4:**231–280.
3. Brinster RL: **Male germline stem cells: from mice to men.** *Science* 2007, **316:**404–405.
4. Spradling A, Fuller MT, Braun RE, Yoshida S: **Germline stem cells.** *Cold Spring Harb Perspect Biol* 2011, **3:**a002642.
5. Waldeyer W: *Eierstock und Ei [Ovary and egg].* Leipzig: Engelmann; 1870.
6. Allen E: **Ovogenesis during sexual maturity.** *Am J Anat* 1923, **31:**439–481.
7. Zuckerman S: **The number of oocytes in the mature ovary.** *Rec Prog Horm Res* 1951, **6:**63–108.
8. Johnson J, Canning J, Kaneko T, Pru JK, Tilly JL: **Germline stem cells and follicular renewal in the postnatal mammalian ovary.** *Nature* 2004, **428:**145–150.
9. Zou K, Yuan Z, Yang Z, Luo H, Sun K, Zhou L, Xiang J, Shi L, Yu Q, Zhang Y, Hou R, Wu J: **Production of offspring from a germline stem cell line derived from neonatal ovaries.** *Nat Cell Biol* 2009, **11:**631–636.
10. Pacchiarotti J, Maki C, Ramos T, Marh J, Howerton K, Wong J, Pham J, Anorve S, Chow YC, Izadyar F: **Differentiation potential of germ line stem cells derived from the postnatal mouse ovary.** *Differentiation* 2010, **79:**159–170.
11. Zou K, Hou L, Sun K, Xie W, Wu J: **Improved efficiency of female germline stem cell purification using fragilis-based magnetic bead sorting.** *Stem Cells Dev* 2011, **20:**2197–2204.
12. Hu Y, Bai Y, Chu Z, Wang J, Wang L, Yu M, Lian Z, Hua J: **GSK3 inhibitor-BIO regulates proliferation of female germline stem cells from the postnatal mouse ovary.** *Cell Prolif* 2012, **45:**287–298.
13. White YA, Woods DC, Takai Y, Ishihara O, Seki H, Tilly JL: **Oocyte formation by mitotically active germ cells purified from ovaries of reproductive-age women.** *Nat Med* 2012, **18:**413–421.
14. Li R, Albertini DF: **The road to maturation: somatic cell interaction and self-organization of the mammalian oocyte.** *Nat Rev Mol Cell Biol* 2013, **14:**141–152.
15. Anckaert E, De Rycke M, Smitz J: **Culture of oocytes and risk of imprinting defects.** *Hum Reprod Update* 2013, **19:**52–66.
16. Xie T, Spradling AC: **A niche maintaining germ line stem cells in the Drosophila ovary.** *Science* 2000, **290:**328–330.
17. Gilboa L, Lehmann R: **Repression of primordial germ cell differentiation parallels germ line stem cell maintenance.** *Curr Biol* 2004, **14:**981–986.
18. Nakamura S, Kobayashi K, Nishimura T, Higashijima S, Tanaka M: **Identification of germline stem cells in the ovary of the teleost medaka.** *Science* 2010, **328:**1561–1563.
19. Draper BW, McCallum CM, Moens CB: **nanos1 is required to maintain oocyte production in adult zebrafish.** *Dev Biol* 2007, **305:**589–598.
20. David GF, Anand Kumar TC, Baker TG: **Uptake of tritiated-thymidine by primordial germinal cells in ovaries of adult slender loris.** *J Reprod Fertil* 1974, **41:**447.
21. Duke KL: **Ovogenetic activity of fetal-type in ovary of adult slow loris Nycticebus coucang.** *Folia Primatol* 1967, **7:**150.
22. Telfer EE: **Germline stem cells in the postnatal mammalian ovary: a phenomenon of prosimian primates and mice?** *Reprod Biol Endocrinol* 2004, **2:**24.
23. Zuckerman S: *Beyond the Ivory Tower: The Frontiers of Public and Private Science.* New York: Taplinger Publishing Company; 1971.
24. Faddy MJ, Telfer E, Gosden RG: **The kinetics of pre-antral follicle development in ovaries of CBA/Ca mice during the first 14 weeks of life.** *Cell Tissue Kinet* 1987, **20:**551–560.
25. Johnson J, Bagley J, Skaznik-Wikiel M, Lee HJ, Adams GB, Niikura Y, Tschudy KS, Tilly JC, Cortes ML, Forkert R, Spitzer T, Iacomini J, Scadden DT, Tilly JL: **Oocyte generation in adult mammalian ovaries by putative germ cells in bone marrow and peripheral blood.** *Cell* 2005, **122:**303–315.
26. Telfer EE, Gosden RG, Byskov AG, Spears N, Albertini D, Andersen CY, Anderson R, Braw-Tal R, Clarke H, Gougeon A, McLaughlin E, McLaren A, McNatty K, Schatten G, Silber S, Tsafriri A: **On regenerating the ovary and generating controversy.** *Cell* 2005, **122:**821–822.
27. Byskov AG, Faddy MJ, Lemmen JG, Andersen CY: **Eggs forever?** *Differentiation* 2005, **73:**438–446.
28. Bukovsky A, Svetlikova M, Caudle MR: **Oogenesis in cultures derived from adult human ovaries.** *Reprod Biol Endocrinol* 2005, **3:**17.
29. Parte S, Bhartiya D, Telang J, Daithankar V, Salvi V, Zaveri K, Hinduja I: **Detection, characterization, and spontaneous differentiation in vitro of very small embryonic-like putative stem cells in adult mammalian ovary.** *Stem Cells Dev* 2011, **20:**1451–1464.
30. Virant-Klun I, Skutella T, Hren M, Gruden K, Cvjeticanin B, Vogler A, Sinkovec J: **Isolation of small SSEA-4-positive putative stem cells from the ovarian surface epithelium of adult human ovaries by two different methods.** *Biomed Res Int* 2013, **2013:**690415.
31. Virant-Klun I, Zech N, Rozman P, Vogler A, Cvjeticanin B, Klemenc P, Malicev E, Meden-Vrtovec H: **Putative stem cells with an embryonic character isolated from the ovarian surface epithelium of women with no naturally present follicles and oocytes.** *Differentiation* 2008, **76:**843–856.
32. Abbott A: **Doubt cast over tiny stem cells.** *Nature* 2013, **499:**390.
33. Antonio-Rubio NR, Porras-Gomez TJ, Moreno-Mendoza N: **Identification of cortical germ cells in adult ovaries from three phyllostomid bats: Artibeus jamaicensis, Glossophaga soricina and Sturnira lilium.** *Reprod Fertil Dev* 2013, **25:**825–836.
34. Rasweiler JJ 4th: **Reproduction in the long-tongued bat, Glossophaga soricina. I. Preimplantation development and histology of the oviduct.** *J Reprod Fertil* 1972, **31:**249–262.
35. Vermande-Van Eck GJ: **Neo-ovogenesis in the adult monkey - consequences of atresia of ovocytes.** *Anat Rec* 1956, **125:**207–224.
36. Bristol-Gould SK, Kreeger PK, Selkirk CG, Kilen SM, Mayo KE, Shea LD, Woodruff TK: **Fate of the initial follicle pool: empirical and mathematical evidence supporting its sufficiency for adult fertility.** *Dev Biol* 2006, **298:**149–154.
37. Wallace WH, Kelsey TW: **Human ovarian reserve from conception to the menopause.** *PloS One* 2010, **5:**e8772.
38. Kerr JB, Duckett R, Myers M, Britt KL, Mladenovska T, Findlay JK: **Quantification of healthy follicles in the neonatal and adult mouse ovary: evidence for maintenance of primordial follicle supply.** *Reproduction* 2006, **132:**95–109.
39. Lei L, Spradling AC: **Female mice lack adult germ-line stem cells but sustain oogenesis using stable primordial follicles.** *Proc Natl Acad Sci U S A* 2013, **110:**8585–8590.
40. Reizel Y, Itzkovitz S, Adar R, Elbaz J, Jinich A, Chapal-Ilani N, Maruvka YE, Nevo N, Marx Z, Horovitz I, Wasserstrom A, Mayo A, Shur I, Benayahu D, Skorecki K, Segal E, Dekel N, Shapiro E: **Cell lineage analysis of the mammalian female germline.** *PLoS Genet* 2012, **8:**e1002477.
41. Woods DC, Telfer EE, Tilly JL: **Oocyte family trees: old branches or new stems?** *PLoS Genet* 2012, **8:**e1002848.
42. Henderson SA, Edwards RG: **Chiasma frequency and maternal age in mammals.** *Nature* 1968, **218:**22–28.
43. Bhartiya D, Sriraman K, Parte S, Patel H: **Ovarian stem cells: absence of evidence is not evidence of absence.** *J Ovarian Res* 2013, **6:**65.
44. Zhao R, Xuan Y, Li X, Xi R: **Age-related changes of germline stem cell activity, niche signaling activity and egg production in Drosophila.** *Aging Cell* 2008, **7:**344–354.
45. Pan L, Chen SY, Weng CJ, Call G, Zhu DX, Tang H, Zhang N, Xie T: **Stem cell aging is controlled both intrinsically and extrinsically in the Drosophila ovary.** *Cell Stem Cell* 2007, **1:**458–469.
46. Signer RA, Morrison SJ: **Mechanisms that regulate stem cell aging and life span.** *Cell Stem Cell* 2013, **12:**152–165.
47. Niikura Y, Niikura T, Tilly JL: **Aged mouse ovaries possess rare premeiotic germ cells that can generate oocytes following transplantation into a young host environment.** *Aging (Alb any NY)* 2009, **1:**971–978.
48. Park ES, Woods DC, Tilly JL: **Bone morphogenetic protein 4 promotes mammalian oogonial stem cell differentiation via Smad1/5/8 signaling.** *Fertil Steril* 2013, **100:**1468–1475. e1462.
49. Lawson KA, Dunn NR, Roelen BA, Zeinstra LM, Davis AM, Wright CV, Korving JP, Hogan BL: **Bmp4 is required for the generation of primordial germ cells in the mouse embryo.** *Genes Dev* 1999, **13:**424–436.
50. Ying Y, Liu XM, Marble A, Lawson KA, Zhao GQ: **Requirement of Bmp8b for the generation of primordial germ cells in the mouse.** *Mol Endocrinol* 2000, **14:**1053–1063.

51. Childs AJ, Kinnell HL, Collins CS, Hogg K, Bayne RA, Green SJ, McNeilly AS, Anderson RA: **BMP signaling in the human fetal ovary is developmentally regulated and promotes primordial germ cell apoptosis.** *Stem Cells* 2010, **28:**1368–1378.
52. Zhang Y, Yang Z, Yang Y, Wang S, Shi L, Xie W, Sun K, Zou K, Wang L, Xiong J, Xiang J, Wu J: **Production of transgenic mice by random recombination of targeted genes in female germline stem cells.** *J Mol Cell Biol* 2011, **3:**132–141.
53. Telfer EE, McLaughlin M, Ding C, Thong KJ: **A two-step serum-free culture system supports development of human oocytes from primordial follicles in the presence of activin.** *Hum Reprod* 2008, **23:**1151–1158.
54. McLaughlin M, Telfer EE: **Oocyte development in bovine primordial follicles is promoted by activin and FSH within a two-step serum-free culture system.** *Reproduction* 2010, **139:**971–978.
55. Anderson RA, McLaughlin M, Woods DC, Tilly JL, Telfer EE: **Evaluation of oogonial stem cells and neo-oogenesis in ovaries of girls and women with Turner syndrome: a pilot study.** *Hum Reprod* 2013, **28:**i52–i55.

10.1186/scrt487
Cite this article as: Dunlop *et al.*: Ovarian germline stem cells. *Stem Cell Research & Therapy* 2014, 5:98

Scandinavian Journal of Clinical & Laboratory Investigation, 2014; 74(Suppl 244): 13–17

informa
healthcare

ORIGINAL ARTICLE

The regulation and assessment of follicular growth

CHERYL E. DUNLOP & RICHARD A. ANDERSON

MRC Centre for Reproductive Health, University of Edinburgh, UK

Abstract

Folliculogenesis is the process by which waves of small primordial follicles possessing immature oocytes are recruited to undergo development into large antral follicles, with one then being selected for ovulation of a fully competent oocyte. Folliculogenesis can be divided into three stages: follicle recruitment, selection and ovulation, and has two phases: the initial gonadotrophin-independent phase and the later gonadotrophin-dependent phase. It involves an elaborate array of biochemical signalling factors, both stimulatory and inhibitory, and the regulation of follicle growth relies on these being tightly controlled. Their increasing understanding allows reproductive biologists to attempt manipulation of folliculogenesis, which can be useful in clinical areas such as assisted reproduction and contraception. The rising average age of childbearing in many developed countries is bringing an additional focus on the importance of assessing a woman's non-growing follicular pool; i.e. her ovarian reserve. This review examines the important regulatory players in the different stages of folliculogenesis and describes some of the currently available measures of ovarian reserve.

Key Words: *Anti-Müllerian hormone, folliculogenesis, non-growing follicular pool, ovarian reserve*

Introduction

The follicle, consisting of an oocyte surrounded by supporting somatic cells, is fundamental to the ovary's role as a reproductive organ. Oocytes are formed in the developing fetus when proliferating primordial germ cells enter meiosis from the 11th week of gestation. The earliest follicular structures, the primordial follicles, consist of an oocyte halted in prophase I of meiosis surrounded by one layer of squamous granulosa cells. They arise once oocytes recruit pre-granulosa cells after around 18 weeks of gestation. Despite there being approximately 7×10^6 oogonia in fetal life, newborn girls have only 1 million primordial follicles; a figure that continues to fall during early life such that females enter their reproductive years with a pool of around 300,000 primordial follicles. The vast majority of these follicles are destined to become atretic. At the start of every menstrual cycle, a cohort, or 'wave', of primordial follicles is recruited to grow, with only one (in monovular species, such as humans and ruminants) or several (in polyovular species, such as mice) follicles achieving dominance by mid-cycle with subsequent release of their now mature and fertilizable, oocyte during ovulation. A myriad of factors, acting at the autocrine, paracrine and endocrine level, are involved in the complex process of folliculogenesis, which comprises follicle recruitment from the primordial pool, dominant follicle selection and, finally, ovulation. This review will examine these three stages, outlining the important biochemical players involved. The methods of assessing follicle growth will also be described.

Initiation of follicular growth

The activation of primordial follicle growth is regulated by a fine balance between stimulatory and inhibitory factors. It is this balance that helps determine the length of a woman's reproductive lifespan and understanding of its regulation is thus a key goal in reproductive biology. Once activated, primordial follicles become primary follicles (an oocyte surrounded by one layer of cuboidal granulosa cells) and then secondary follicles (with multiple layers of granulosa cells and an outermost theca cell layer) which subsequently develop a fluid filled antral cavity. Selection for ovulation occurs

Correspondence: Richard A. Anderson, MRC Centre for Reproductive Health, University of Edinburgh, Edinburgh, Scotland, UK. E-mail: Richard.Anderson@ed.ac.uk

ISSN 0036-5513 print/ISSN 1502-7686 online
DOI: 10.3109/00365513.2014.936674

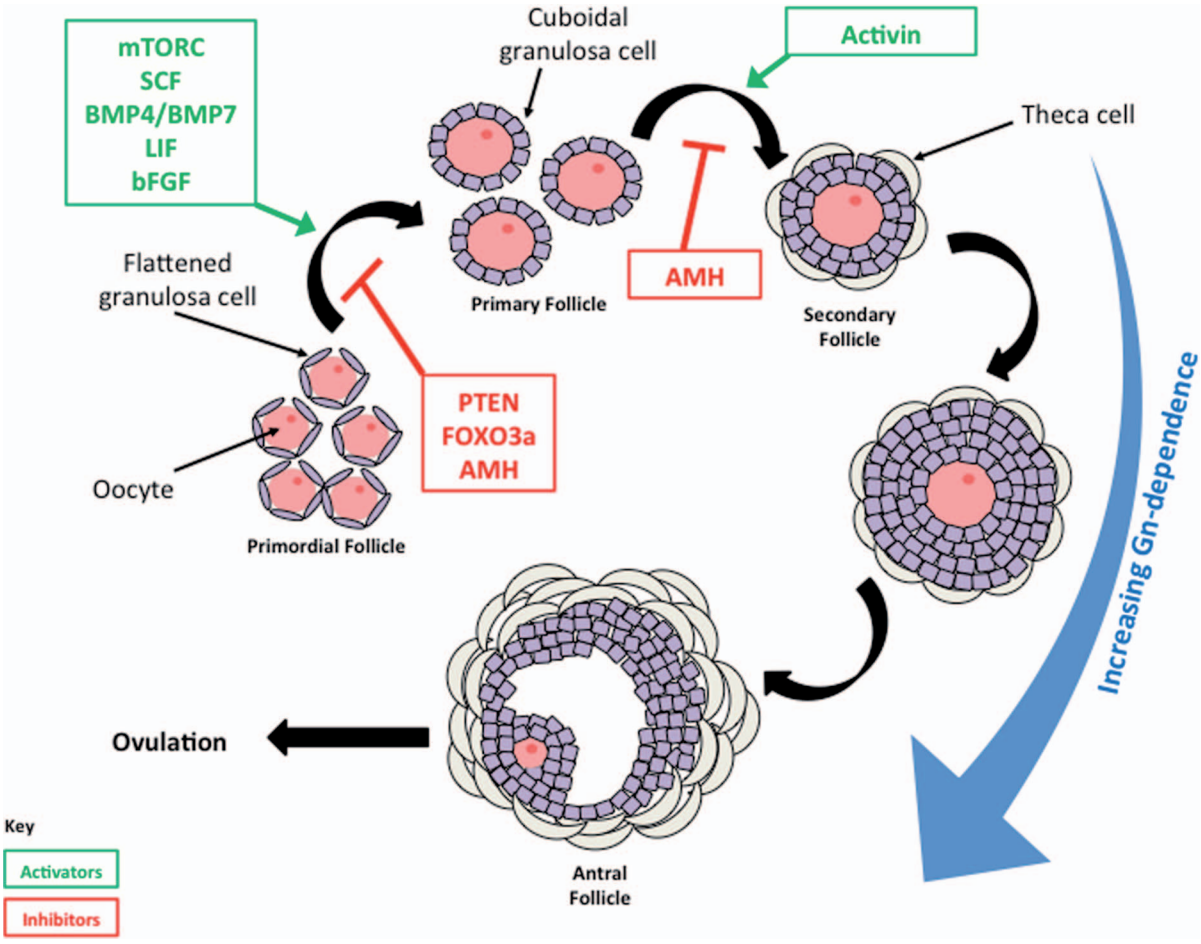


Figure 1. A summary of folliculogenesis. The primordial follicle pool develops into primary then secondary follicles independently of gonadotrophins, but the process is closely regulated by activating (green box) and inhibitory (red box) factors. Growth from a secondary follicle to an antral follicle is increasingly gonadotrophin-dependent, with ovulation being entirely gonadotrophin-dependent.

following further growth and with increasing gonadotropin dependence, rather than the gonadotropin sensitivity of earlier stages (Figure 1).

A critical pathway identified to be involved in the regulation of the initiation of primordial follicle growth is the phosphatidylinositide 3-kinase (PI3K) pathway, located within the oocyte. When this intracellular signalling pathway is activated by growth factors such as stem cell factor (SCF; also known as Kit ligand), a cytokine produced by the granulosa cells of the primordial follicle, an increase in PI3K results in increased phosphorylation of Akt, a serine/threonine-specific protein kinase, with the end result being increased cell survival and proliferation and inhibition of apoptosis. Follicular activation can also be stimulated via this pathway by the mammalian target of rapamycin complex (mTORC). The role of mTORC in human follicular activation has been shown by demonstrating that treatment of immature follicles with rapamycin, an inhibitor of mTORC, can lead to oocyte death [1].

The PI3K pathway is kept in control by inhibitory factors, including phosphatase and tensin homolog (PTEN) and the transcription factor forkhead box O3a (FOXO3a). PTEN inhibits the stimulation of Akt and therefore suppresses cell proliferation. Consequently, PTEN null mice demonstrate premature activation of the primordial follicle pool, thus shortening the reproductive lifespan considerably [2]. This is oocyte specific, as PTEN deficiency in granulosa cells does not induce this phenotype. FOXO3a, a member of the forkhead family of transcription factors, is another important downstream component to the PI3K pathway. Like PTEN, FOXO3a inhibits follicular activation and thus knock-out mice also demonstrate premature ovarian insufficiency [3].

Recently, another intracellular signalling pathway, the Hippo signalling pathway, has been suggested as potentially having a role in follicle activation [4]. The Hippo pathway is a highly conserved kinase cascade, activated by cell membrane-bound regulators in areas of high cell density. Activation leads to phosphorylation and inactivation of a cell proliferation transcription factor called yes-associated protein (YAP). Therefore, in cell dense tissue, cell growth and proliferation is impeded, whilst in areas of low cell density, the pathway remains inactivated and YAP promotes cell growth: this mechanism has been implicated in

determining organ size, and may explain why fragmentation of ovarian cortex, and hence disruption of the Hippo pathway, leads to increased initiation of follicle growth [4].

Several other factors have been shown to activate primordial follicles to form primary follicles [5]. Bone morphogenetic proteins 4 and 7 (BMP4 and BMP7) are members of the transforming growth factor β (TGFβ) family, with proven roles in early follicular development. Furthermore, BMP4 is necessary for the survival of oocytes. Leukaemia inhibitory factor (LIF), a granulosa cell-secreted cytokine, and fibroblastic growth factor (bFGF), produced by the oocyte, also appear to stimulate the transition of primordial follicles to primary follicles in various species [5].

Conversely there are important inhibitory factors present in the ovary to control the rate of primordial follicle activation, one of which is anti-Müllerian hormone (AMH). Like the BMPs, AMH is a member of the TGFβ superfamily; however, a key role appears to be suppression of primordial follicle recruitment [6]. AMH is produced by the granulosa cells of preantral and small antral follicles, with a sharp decline in larger antral follicles [7]. It is possible that AMH produced by the cohort of growing preantral and early antral follicles inhibit the growth of their neighbouring primordial follicles, thereby restraining the number of follicles activated at any one time. In addition to its direct inhibitory role in early follicle development, AMH can also suppress activation indirectly, by reducing the expression of SCF and bFGF [5].

After activation from primordial follicles to primary follicles, the next step is development into secondary, or pre-antral, follicle stages. This process requires further oocyte growth and proliferation of the granulosa cells. Once again, members of the TGFβ superfamily play a role, with the dimeric protein activin enhancing granulosa cell proliferation [8]. At this point, follicular growth is gonadotrophin-sensitive rather than dependent; activin also promotes the responsiveness of granulosa cells to follicle-stimulating hormone (FSH) [8], and, consequently, further follicular growth relies on gonadotrophins. Growth differentiation factor 9 (GDF9) and BMP15 are both expressed by the oocyte and are essential in the development of follicles past the primary stage [9]. They work synergistically to regulate cell proliferation within the follicle and regulate SCF expression. AMH has a further inhibitory role at this stage of folliculogenesis, by reducing FSH-sensitivity. The down-regulation of AMH expression as follicles grow coincides with an equally sharp upregulation of aromatase expression, and thus the capacity of the follicle to make estradiol [7]. AMH is thus not only involved in controlling the rate at which follicles leave the primordial pool, but it may play a role in the selection of the dominant follicle. While these growth factors have increasingly defined roles, there are likely to be other, as yet, unrecognized pathways of key importance.

A crucial aspect to the whole process is the ability for the oocyte and its surrounding somatic cells to communicate with each other. It is now clear that this communication is bi-directional, via gap junctions and transzonal projections, which allow essential molecules to pass between the oocyte and granulosa cells (reviewed in [10]).

Follicle selection and ovulation

As follicles grow, they become progressively more dependent on gonadotrophins for continued development and survival. FSH promotes granulosa cell proliferation and differentiation, allowing the follicle to increase in size. By the time the follicle forms an antrum, from follicular fluid produced by the granulosa cells, it is entirely FSH-dependent for further development. Both the granulosa cells and theca cells express gonadotrophin receptors and become responsible for sex steroidogenesis, with theca cells becoming LH responsive (thus producing androgen) and granulosa cells responding to FSH (and converting theca cell-derived androgen to oestradiol by aromatization: the two-cell, two-gonadotrophin hypothesis). By now, granulosa cells can be compartmentalized into two groups: cumulus cells surrounding the oocyte and promoting its maturation, and mural granulosa cells around the inner aspect of the follicle, producing sex steroids. The follicle destined for when ovulation occurs is now ~10–12 mm in diameter and can be accurately monitored by ultrasound.

Subsequent ovulation is triggered by a surge of LH and an antral follicle will only become preovulatory if both its granulosa cells and theca cells express LH receptors at the time of this surge. It has become clear that the effect of the LH surge is mediated through EGF family members including epiregulin and amphiregulin produced by mural granulosa cells and acting on the cumulus [11]. Most mammals control their ovulation rate in order to regulate the number of offspring they can conceive at one time. In humans, this requires that only one growing follicle attains 'dominance', whilst the rest become 'subordinate' and, eventually, atretric. The importance of the oocyte in regulating this process is demonstrated by the effect of heterozygosity for mutations in BMP15 and GDF9 in sheep which can result in higher ovulation rates, whereas homozygosity leads to sterility [12].

The basis for the model for the regulation of ovulation rate was first proposed almost 30 years ago [13]. It is thought that, during a follicular wave, there is a figurative 'gate' or 'window' of time during which FSH concentrations are above the threshold necessary for large, gonadotrophin-dependent follicles to escape atresia. The length of the FSH 'window' thereby dic-

tates how many follicles become ovulatory: one would hypothesize that monovular species have a short window, thus allowing only one follicle to undergo ovulation following the necessary LH surge.

Clearly, folliculogenesis is complicated and not yet fully understood. However, could some of the major players involved be useful clinically in assessing the follicular pool?

Assessment of the follicular pool

It is widely believed that women are born with a finite supply of oocytes (although this has been challenged in the last decade (reviewed in [14]). As such, the size of the non-growing follicular (NGF) pool determines the length of a woman's reproductive lifespan. For clinical purposes, it is useful to know the size of a woman's available follicular pool to aid assessment and management of infertility. Furthermore, the increasing trend of delaying having children in women in western societies indicates that accurate prediction of ovarian reserve is important, not only medically, but sociologically, as women seek to find out how long they have left on their 'biological clock'. These two pools should be distinguished: the term 'ovarian reserve' is often used in this context although more correctly should be reserved for the primordial follicle pool. What is often meant is the potential ovarian response to exogenous gonadotrophins (most commonly in superovulation for IVF), which consists of follicles already at the antral stage of development. At present there are no known biomarkers of the primordial follicle pool, and the available markers have, at best, indirect relationships with the number of primordial follicles in the ovary based on the assumption that the size of the pool of follicles that are potentially recruitable is related to the primordial follicle number, allowing prediction of reproductive lifespan. Histological analysis of primordial follicle number has been correlated with ultrasound and serum assessment of ovarian reserve markers [15] showing that there are indeed useful relationships, but the indirectness of these relationships must always be borne in mind in interpretation. This has been recently demonstrated in an analysis of AMH in children with newly diagnosed cancer, which showed that AMH was generally low compared to age-matched controls, and correlated negatively with markers of ill health such as C reactive protein [16].

One physical method of evaluating the ovarian reserve is measurement of the antral follicle count (AFC) by transvaginal ultrasonography (TVUSS). The AFC is the number of follicles less than 10 mm in diameter in the early follicular phase of the menstrual cycle and has a close inverse correlation with age. However, AFC shows some variation from one menstrual cycle to the next and there is inter-observer variability by the sonographer. Additionally improvements in ultrasound technology have had an impact on image quality and thus follicle recognition: this is of importance where the AFC is used for diagnostic purposes, e.g. in polycystic ovary syndrome [17].

Various serum biomarkers have also been proposed for the prediction of the size of the remaining follicular pool. Three hormones that have been assessed as biomarkers are FSH, inhibin B and AMH; however, the 'perfect' biomarker is yet to be found. Early follicular phase FSH has been the most extensively used biomarker to date as it has long been measurable and indicates ovarian feedback on the hypothalamic-pituitary axis. As women age and their ovarian reserve declines, FSH concentrations rise. The major drawback is the marked cycle-to-cycle variation in FSH, as well as the need for the sample to be taken in the early follicular phase.

Inhibin B is produced by the granulosa cells of small antral follicles and is a key physiological inhibitor of FSH secretion, limiting the inter-cycle FSH rise. There was considerable interest in inhibin B as a predictor of ovarian response in assisted reproduction but it has now largely been replaced by measurement of AMH.

AMH has now been used in clinical practice for some years for the assessment of response to superovulation regimens used in *in vitro* fertilization (IVF), and its role and potential value is increasingly recognized [18]. It is useful as a predictor of those likely to respond poorly, but unlike FSH can also identify women at high risk of ovarian hyperstimulation. AMH has the advantage of low inter- and intra-cycle variability so can be measured at any time of the menstrual cycle. It is also being explored as a predictor of time to menopause, provided a woman's age is accounted for when interpreting the measurement [19]. It is, however, affected by suppressed gonadotrophin levels, with lower AMH concentrations seen during pregnancy and long-term gonadotrophin-releasing hormone (GnRH) analogue treatment. In addition, there is a pressing need for standardization of AMH quantification between the available commercial assays [18].

While AMH shows much promise as a serum biomarker, in general it is of very similar predictive value as AFC and the clinical situation will therefore at present determine which test is preferred. There remains a need for more biomarkers for the earliest stages of follicle number, and ultimately of primordial follicle number. Increasing molecular analysis of follicle populations using human samples may well provide insights of value in this quest [20].

Conclusion

It is evident that a multitude of signalling factors are required, at the right concentrations and at the right time, in order for a competent, mature oocyte to be produced on a monthly basis. Furthermore, a fine balance of activating and inhibitory factors is essential to regulate the rate at which follicles are recruited and ovulated. Clinically, knowledge of these factors

is important if we wish to understand the regulation that allows follicles formed before birth to suffice for 50 years of ovarian activity. Additionally, it is necessary for manipulation of folliculogenesis for use in assisted reproductive techniques and new contraceptives. Although AMH is showing promise in the assessment of ovarian reserve, there is still much to learn about the process of folliculogenesis and further research may yet uncover a new, more accurate biomarker of ovarian reserve.

Questions and answers

Q (Villa): Does the relationship between AMH (anti-Müllerian hormone) and follicular growth vary in different pathological conditions?

A (Anderson): There is a little data on this but not much. Recently a group from Rotterdam published a paper in the journal *Human Reproduction*, which showed that at the point of diagnosis, AMH concentrations were lower than they ought to be in children with cancer. Also the concentrations were inversely correlated to CRP concentrations and to hemoglobin concentrations, so the more ill the child, the lower the AMH.

Comment (Carmina): There are also data in patients with Klinefelter's disease and in patients with delayed puberty. AMH is used by pediatric oncologists to differentiate patients who will go on to regular puberty from those who will not.

Comment (Anderson): Related to this is the gonadotropin dependence of AMH. Long-term gonadotropin suppression treatment will lead to decreased AMH.

Comment (Burney): There are other situations in which AMH is decreased such as endometriosis, inflammatory bowel disease and pelvic inflammatory disease.

Q (Burney): Is there any progress towards a reference standard for AMH?

A (Anderson and Beastall): There has been talk about this and moves towards it. There is growing recognition that AMH is a biomarker which is of use and for which there is a future. If it is to be used for clinical purposes, it is essential that assays are good and standardization is a prerequisite for this. There are other possible markers but the data on them is not robust and the evidence is sometimes indirect.

Q (Ballieux): My professor in Obstetrics and Gynaecology says that ultrasound is better than AMH which is thus unnecessary. Is AMH useful for patient care or for research?

A (Anderson): Ultrasound examinations are expensive and in primary care a blood test is much more useful. AMH concentration measurements are likely to become of much more clinical use in the future.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

The authors' work in this field is supported by MRC grant G1100357.

References

[1] McLaughlin M, Patrizio P, Kayisli U, et al. mTOR kinase inhibition results in oocyte loss characterized by empty follicles in human ovarian cortical strips cultured in vitro. Fertil Steril 2011;96:1154–9e1.

[2] Reddy P, Liu L, Adhikari D, et al. Oocyte-specific deletion of Pten causes premature activation of the primordial follicle pool. Science 2008;319:611–3.

[3] Castrillon DH, Miao L, Kollipara R, et al. Suppression of ovarian follicle activation in mice by the transcription factor Foxo3a. Science 2003;301:215–8.

[4] Kawamura K, Cheng Y, Suzuki N, et al. Hippo signaling disruption and Akt stimulation of ovarian follicles for infertility treatment. Proc Natl Acad Sci USA 2013;110:17474–9.

[5] Skinner MK. Regulation of primordial follicle assembly and development. Hum Reprod Update 2005;11:461–71.

[6] Durlinger AL, Gruijters MJ, Kramer P, et al. Anti-Mullerian hormone inhibits initiation of primordial follicle growth in the mouse ovary. Endocrinology 2002;143:1076–84.

[7] Jeppesen JV, Anderson RA, Kelsey TW, et al. Which follicles make the most anti-Mullerian hormone in humans? Evidence for an abrupt decline in AMH production at the time of follicle selection. Mol Hum Reprod 2013;19:519–27.

[8] Knight PG, Satchell L, Glister C. Intra-ovarian roles of activins and inhibins. Mol Cell Endocrinol 2012;359:53–65.

[9] Gilchrist RB, Lane M, Thompson JG. Oocyte-secreted factors: regulators of cumulus cell function and oocyte quality. Hum Reprod Update 2008;14:159–77.

[10] Albertini DF, Combelles CM, Benecchi E, et al. Cellular basis for paracrine regulation of ovarian follicle development. Reproduction 2001;121:647–53.

[11] Hsieh M, Lee D, Panigone S, et al. Luteinizing hormone-dependent activation of the epidermal growth factor network is essential for ovulation. Mol Cell Biol 2007;27:1914–24.

[12] Scaramuzzi RJ, Baird DT, Campbell BK, et al. Regulation of folliculogenesis and the determination of ovulation rate in ruminants. Reprod Fertil Dev 2011;23:444–67.

[13] Baird DT. A model for follicular selection and ovulation: lessons from superovulation. J Steroid Biochem 1987;27:15–23.

[14] Hanna CB, Hennebold JD. Ovarian germline stem cells: an unlimited source of oocytes? Fertil Steril 2014;101:20–30

[15] Hansen KR, Hodnett GM, Knowlton N, et al. Correlation of ovarian reserve tests with histologically determined primordial follicle number. Fertil Steril 2011;95:170–5.

[16] van Dorp W, van den Heuvel-Eibrink MM, de Vries AC, et al. Decreased serum anti-Mullerian hormone levels in girls with newly diagnosed cancer. Hum Reprod 2014;29:337–42.

[17] Dewailly D, Gronier H, Poncelet E, et al. Diagnosis of polycystic ovary syndrome (PCOS): revisiting the threshold values of follicle count on ultrasound and of the serum AMH level for the definition of polycystic ovaries. Hum Reprod 2011;26:3123–9.

[18] Dewailly D, Andersen CY, Balen A, et al. The physiology and clinical utility of anti-Mullerian hormone in women. Hum Reprod Update 2014;20:370–385. doi: 10.1093/humupd/dmt062 [Epub ahead of print]

[19] Freeman EW, Sammel MD, Lin H, et al. Anti-Mullerian hormone as a predictor of time to menopause in late reproductive age women. J Clin Endocrinol Metab 2012;97:1673–80.

[20] Kristensen SG, Andersen K, Clement CA, et al. Expression of TGF-beta superfamily growth factors, their receptors, the associated SMADs and antagonists in five isolated size-matched populations of pre-antral follicles from normal human ovaries. Mol Hum Reprod 2014;20:293–308.